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IN VITRO STUDIES OF ASPECTS OF THE METABOLISM OF SODIUM BY HUMAN ERYTHROCYTES USING SODIUM[22]

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NEW ORLEANS, LA.

INTEREST in the mechanisms of sodium metabolism in body cells has been increased by observations made in recent years. For instance, it has been shown that sodium enters cells in significant quantities when potassium leaves these cells as the result of disease. In man, such cellular sodium increases are associated with an alkalosis.[1-4] Again, evidence has accumulated to indicate that salt-active adrenal steroids may possess significant influence upon the water and electrolyte metabolism of cells in organs other than those involved in electrolyte excretion,[5-7] and these steroids may therefore be involved in such shifts of base between cells and extracellular fluid. This may have clinical importance in syndromes such as congestive heart failure where some of the unknown processes in pathogenesis may involve modifications in electrolyte metabolism in cells.[8, 9] Coupled with these observations is evidence, obtained with radioactive isotopes, suggesting that the intracellular sodium of the body is of considerable quantity, and that this sodium is continuously exchanging with the extracellular sodium.[10]

Erythrocytes readily lend themselves to in vitro study of certain aspects of the basic processes and principles of cellular sodium metabolism because they can be removed from the body and maintained under satisfactory conditions. There is no absolute assurance, of course, that such isolated cells maintain the same mechanisms of sodium metabolism existent in vivo or possess the same mechanisms of sodium metabolism found in other cells of the body. Their in vitro state is unlikely to differ qualitatively from a physiological one, only quantitatively, if to a measurable extent at all. The erythrocyte may permit some evaluation of general cellular sodium metabolism, since its content of sodium and potassium approximates that of human cells in general, and the maintenance of a low sodium and high potassium level involves active metabolism by the cell, especially glycolysis.[11, 12]

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*Life Insurance Medical Research Fellow 1950-52.

TABLE I. SUMMARY OF ALL MEASUREMENTS OF RED CELL SODIUM EXCHANGE

EXPERIMENT NO.	DONOR	$T_{1/2}$ HOURS	RAPIDLY EXCHANGING R.B.C. Na MEQ./L. R.B.C.	TOTAL RED CELL Na (MEQ./L. R.B.C.)	EXCHANGE RATE MEQ./L. R.B.C./HR.	EXCHANGE RATE % RAPIDLY EXCHANGING SODIUM	NOTES
1	S. A. T.	1.0	4.7	7.7	3.2	68	Standard conditions
2	A. F.	1.0	5.7	8.9	3.9	69	Standard conditions
3	G. B.	1.5	6.2	9.5	2.9	46	Standard conditions
4	R. M.	1.1	5.5	7.4	3.5	63	Standard conditions except done in multiple flasks
5	W. L.	2.4	6.0	5.5	1.7	29	Na^{22} not added until blood kept in vitro for 18 hr.
6	W. L.	1.0	5.3	7.6	3.7	69	Standard conditions
7	W. L.		5.4	5.1			Uptake rate not measured
8	R. M.	0.9	4.9	5.4	3.8	78	Standard conditions
9	A. F.	3.0	6.5	6.5	1.5	23	Done at 28° C.
10	A. F.	1.2	6.8	7.3	3.9	57	K was raised in the plasma by 10 meq./L. after 40 min. of incubation without grossly affecting the uptake rate
11	S. A. T.	1.0	6.4	6.3	4.4	69	Standard conditions
12	V. R.	1.3	13.3	14.5	7.1	53	Donor was a Negro female patient with myxedema and congestive heart failure
13	A. G.	1.5	7.9	13.8	3.9	47	Donor was a Negro female patient with pneumonia and pleural effusion
14-a	R.B.C. Plasma A. G. A. G.	2.0	9.1	11.5	3.2	35	The cells of Donor A. G. with "bound" sodium were tested in plasma from Donor W. L. who had no "bound" sodium (b) and vice versa (d)
14-b	A. G. W. L.	1.9	8.1	11.1	2.9	36	
14-c	W. L. W. L.	1.2	5.3	5.1	3.1	59	
14-d	W. L. A. G.	1.0	5.2	5.1	3.6	69	
15	R. M.	3.0	8.2	7.6	1.9	23	Done at 28° C.
16	G. B.	1.4	8.0	8.3	3.9	49	Standard conditions
17-a	R. Z.	1.5	5.5	5.0	2.5	45	Control

TABLE I (CONT'D)

17-b	R. Z.	1.3	5.5	6.1	3.0	55	Lanatoside C added, $9 \cdot 10^{-4}$ mg./ml. of plasma
17-c	R. Z.	1.2	5.1	5.2	2.9	57	Neostigmine added, $4 \cdot 10^{-5}$ mg./ml. of plasma
17-d	R. Z.	1.2	4.9	5.2	2.8	57	Epinephrine added, $2 \cdot 10^{-5}$ mg./ml. of plasma
18-a	R. M.	0.8	5.3	6.1	4.9	92	Control
18-b	R. M.	1.0	5.2	6.2	3.8	73	Pitressin added, 0.13 unit/ml. of plasma
18-c	R. M.	1.1	5.2	6.6	3.3	63	Cortisone added, 0.16 mg./ml. of plasma
18-d	R. M.	1.1	5.3	5.9	3.3	62	DOCA added, 0.06 mg./ml. of plasma
19-a	E. F.	1.8	8.3	7.8	3.2	39	Control
19-b	E. F.	1.8	6.6	7.5	2.6	39	Supernatant plasma removed and 0.15M NaCl with 200 mg. % glucose substituted
19-c	E. F.	2.0	2.5	3.6	0.9	36	Supernatant plasma removed and 0.15M KCl with 200 mg. % glucose substituted

This report is concerned with a study of the sodium within the red cells of a small group of human subjects. The total intracellular sodium was measured with a flame photometer while the amount of rapidly exchanging red cell sodium was determined with Na^{22}. This was done by adding the isotope to the plasma and then following the rise in red cell radioactivity until it had reached a plateau value, usually after four to six hours. This exchanging fraction of red cell sodium is referred to hereafter as the rapidly exchanging sodium, and it is quantitatively compared with the total red cell sodium. The rate of exchange was also studied under standard conditions simulating the in vivo red cell environment. In addition, the influence of variations in temperature, of changes in plasma potassium and sodium concentrations, and of several drugs and chemicals added to the blood was studied.

MATERIALS AND METHODS

Source and Collection of Blood.—Blood was obtained from healthy male laboratory personnel and two hospitalized female patients. These latter two subjects were selected because their erythrocytes were known to have a high sodium content. All subjects did not fast. Blood was drawn from the median basilic vein with stasis through a No. 18 needle into a

syringe containing 10 mg. of heparin without preservative in one ml. of 0.15M aqueous sodium chloride solution per 50 ml. of blood.

Incubation of Blood.—Immediately after venipuncture, the blood was transferred to a paraffined flask where it was exposed to an atmosphere of 6 per cent carbon dioxide, 8 per cent oxygen, and 86 per cent nitrogen at 38° C. The method has been described in detail elsewhere.[13] With one exception, which is noted in Table I, a single flask was used so that all aliquots removed for analysis would contain the same amount of Na^{22}. In those experiments in which drugs were added, incubation was carried out in smaller flasks under air.

Addition of Isotope.—At zero time, one to two ml. of plasma containing a suitable amount of Na^{22} and sufficient glucose to raise the blood level 250 mg. per cent was added to the whole blood and promptly mixed with it.

Isotopes.—One sample of Na^{22} which produced a progressive loss of potassium from the incubated red cells was found to contain copper. By the dithizone technique, the amount of copper present approximated 10^{-5} molar in the whole blood. Despite the copper, the measurements of sodium exchange made with this specimen of Na^{22} showed no differences from those obtained with Na^{22} without copper.

Chemical Analyses.—At appropriate intervals after adding Na^{22}, samples of the whole blood were removed from the flask for analysis. Measurements of cellular and plasma sodium were made by flame photometry, as previously described.[14] In each sample, the cells were separated from the plasma and analyzed directly. The data for red cell sodium were the least reliable, especially in the early experiments before the methods were finally improved.

Measurement of Radioactivity.—Samples of plasma and of red cells were prepared and counted by techniques described previously.[13, 15] The plasma was diluted five times with water before counting. Red cell preparations were made in duplicate or triplicate and counted for periods sufficient to reduce counting variability to below five per cent.

Calculations.—Calculation of the rate of exchange of sodium between red cells and plasma was made by a modification of the method of Sheppard for the two-compartment system.[16] The exchange rate between the two compartments of a closed system is given by the expression:

$$\rho = \frac{0.69(S_1 S_2)}{T_{1/2}\ (S_1 + S_2)}$$

where,

ρ is exchange rate, i.e., the amount of sodium entering or leaving the red cells of a given volume of whole blood per unit time,

$T_{1/2}$ is the time required for the equalization of specific activities in the two compartments to proceed half way, after the tracer has been added to one,

S_1 is the total plasma sodium per unit volume of whole blood, and

S_2 is the total rapidly exchanging red cell sodium per unit volume of whole blood.

Since in all experiments there was over ten times as much plasma sodium as red cell sodium participating in the exchange, and since all the tracer was added to the plasma compartment at zero time, the plasma radioactivity fell less than 10 per cent during the course of the experiment, usually less than 5 per cent. Therefore, the plasma compartment can be considered as an infinite source of sodium at the average plasma specific activity without introducing important errors. With this assumption, the exchange rate is then given by:

$$\rho = \frac{0.69}{T_{1/2}} \cdot S_2$$

$T_{1/2}$ was obtained from a semilogarithmic plot of the difference between the red cell radioactivity at any time and its value when a plateau was reached, i.e., when mixing of exchangeable sodium with the plasma was essentially completed. S_2 cannot be found except by the use of the radioactivity data, since the rapidly exchanging and total sodium contents of the cells may be different. Therefore, S_2 was obtained from the expression:

$$S_2 = \frac{\text{R.B.C. counts/min./L. of R.B.C. at plateau value}}{\text{mean plasma specific activity}}$$

Because the mean plasma specific activity was used, the value for S_2 was somewhat lower than the actual value. For convenience, rate of exchange is expressed in milliequivalents of sodium per liter of red cells per hour.

The plot from which the $T_{\frac{1}{2}}$ was obtained was employed as a partial index of the reliability of the calculated rate of exchange. This was possible because deviations from a straight line indicate technical error, inhomogeneity of rates of exchange, changing rate of exchange, or change in the amount of exchanging sodium.

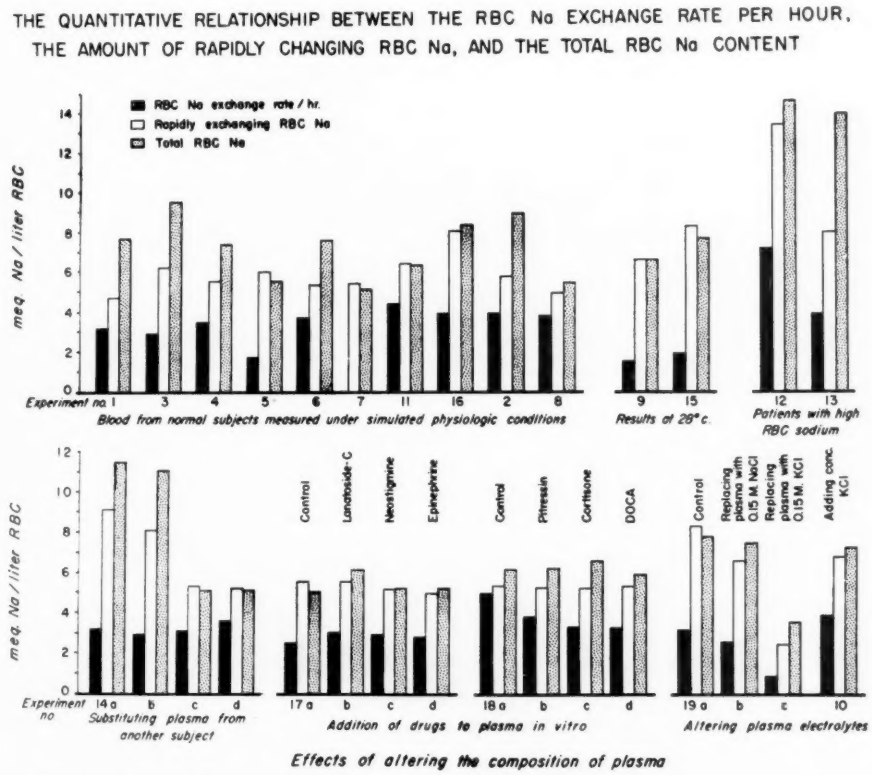
THE QUANTITATIVE RELATIONSHIP BETWEEN THE RBC Na EXCHANGE RATE PER HOUR, THE AMOUNT OF RAPIDLY CHANGING RBC Na, AND THE TOTAL RBC Na CONTENT
RBC Na exchange rate / hr.
Rapidly exchanging RBC Na
Total RBC Na
meq. Na / liter RBC
Experiment no. 1 3 4 5 6 7 11 16 2 8
Blood from normal subjects measured under simulated physiologic conditions
9 15
Results at 28° c.
12 13
Patients with high RBC sodium
Experiment no
14 a b c d
Substituting plasma from another subject
17 a b c d
Control
Lanatoside-C
Neostigmine
Epinephrine
Addition of drugs to plasma in vitro
18 a b c d
Control
Pitressin
Cortisone
DOCA
19 a b c 10
Control
Replacing plasma with 0.15 M. NaCl
Replacing plasma with 0.15 M. KCl
Adding conc. KCl
Altering plasma electrolytes
Effects of altering the composition of plasma

Fig. 1.—The relationship between R.B.C. sodium exchange rate per hour, rapidly exchanging sodium, and total sodium. The experiments were numbered chronologically and their numbers appear in the abscissa. The blood of two subjects was studied at both 28° C. and 38° C. Experiments 2 and 9 were obtained on one subject's blood, and Experiments 8 and 15 on the other's.

RESULTS

The results of all experiments are summarized in Table I. In Fig. 1 the quantitative relationships in each experiment between the total red cell sodium, as determined flame photometrically, the rapidly exchanging sodium, as measured with Na^{22}, and the amount of sodium exchanging per hour are presented.

In one instance, gross hemolysis occurred after less than one hour of incubation of the blood, so that this experiment was not completed. In all other cases, it was possible to calculate satisfactorily the exchange rate and rapidly

exchanging sodium content of the red cells. One requirement necessary for satisfactory calculations was the maintenance of constant sodium content of the red cells for the first four to six hours of incubation. This requirement was approached to varying degrees in the experiments, but was never absolutely

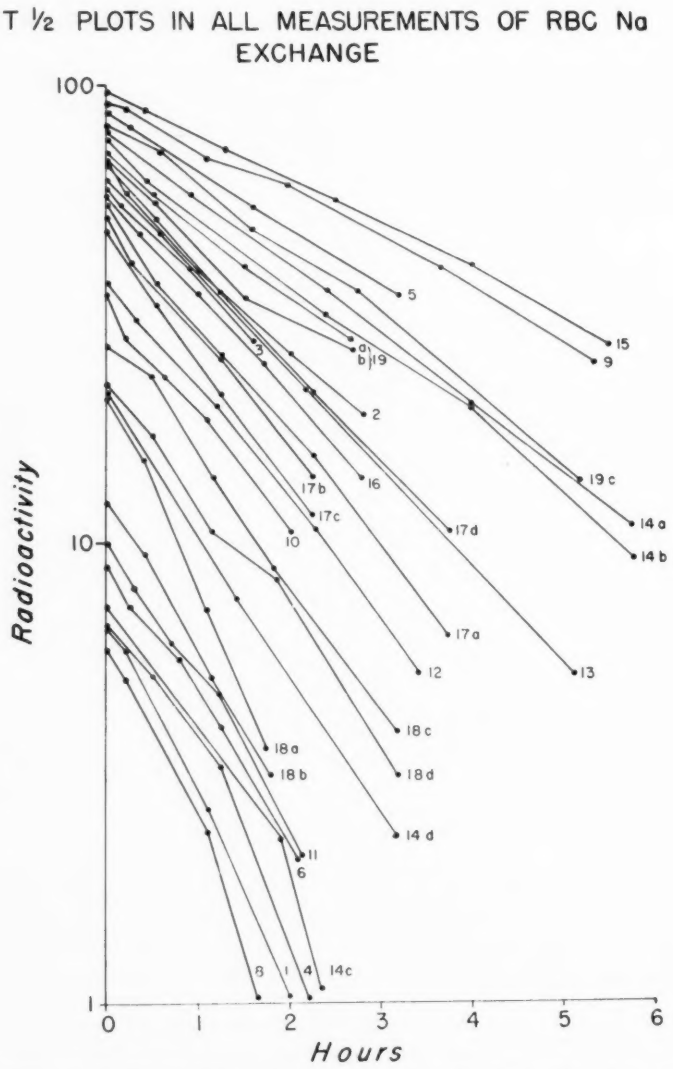

Fig. 2.—$T_{½}$ plots of all experiments. The time course of the logarithm of the difference between red cell radioactivity at the plateau level and at any time is shown.

obtained. This variation in sodium content is reflected in part in the $T_{½}$ plots of each experiment (Fig. 2). As indicated above, if the exchange rate and exchanging sodium content of the red cells are constant, these plots present straight lines. Actually, however, there was a consistent tendency for the

red cell sodium to fall slightly during the early hours of the experiment, and to begin a gradual rise after four to six hours. Since the calculations of the exchange rate are based predominantly on the first two hours, errors due to changing cell sodium content are small.

The results of an experiment on blood from a normal subject (Experiment 11) are presented in detail in Table II and Fig. 3, *A*. This is an instance in which the rapidly exchanging and total sodium contents of the red cells were essentially the same. An experiment on the blood of one of the patients with a high R.B.C. sodium, on the other hand, showed considerable differences between these values (Table III, Experiment 13 and Fig. 3, *B*).

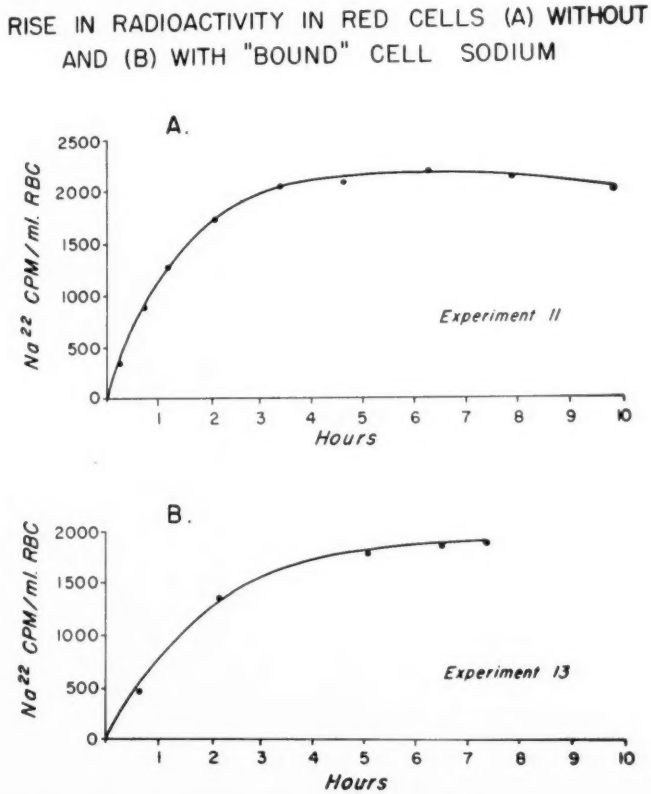

Fig. 3.—The rise in red cell radioactivity after adding Na^{22} to the plasma is shown in a case where rapidly exchanging and total R.B.C. sodium were equal (*A*, Experiment 11) and one in which there was "bound" R.B.C. sodium (*B*, Experiment 13).

Six drugs were tested for possible effects on erythrocyte sodium metabolism by adding them to the incubating blood in the amounts shown in Table I. The results are summarized in Table I and Figs. 1 and 4, *A* and *B*.

The effects of variations in the plasma concentration of sodium and potassium on sodium transfer rate were studied by adding concentrated KCl solution to the plasma after the experiment had begun (Experiment 10, Table

TABLE II. DATA OF EXPERIMENT 11 IN WHICH THE TOTAL RED CELL SODIUM MEASURED FLAME PHOTOMETRICALLY AND THE RAPIDLY EXCHANGING RED CELL SODIUM MEASURED WITH Na^{22} WERE EQUAL

SPECIMEN NUMBER	TIME		HEMATOCRIT	HEMOLYSIS	RADIOACTIVITY IN CTS./MIN./ML.		ELECTROLYTE CONCENTRATIONS IN MEQ./L.			
	HOURS	MINUTES			PLASMA	R.B.C.	PLASMA SODIUM	PLASMA POTASSIUM	R.B.C. POTASSIUM	R.B.C. SODIUM
1.		14	44.7	0	50,400	430	145	3.3	89	6.0
2.		42	44.1	0	50,400	870	143	3.3	87	6.2
3.	1	11	44.3	0	49,900	1270	143	3.3	87	6.4
4.	2	17	44.1	0	48,900	1730	141	3.4	88	6.6
5.	3	24	44.2	0	50,000	2040	143	3.3	86	6.2
6.	4	38	44.0	+	50,300	2080	142	3.3	85	6.3
7.	6	16	44.2	+	47,300	2160	142	3.3	84	5.4
8.	7	56	44.8	++	48,200	2100	145	3.4	87	5.5
9.	9	51	44.8	+++	47,700	2060	145	3.7	87	5.3

TABLE III. DATA OF EXPERIMENT 13 IN WHICH THE TOTAL RED CELL SODIUM MEASURED FLAME PHOTOMETRICALLY WAS 5.9 MEQ./L. HIGHER THAN THE RAPIDLY EXCHANGING SODIUM MEASURED WITH Na^{22}

SPECIMEN NUMBER	TIME		HEMATOCRIT	HEMOLYSIS	RADIOACTIVITY IN CTS./MIN./ML.		ELECTROLYTE CONCENTRATIONS IN MEQ./L.			
	HOURS	MINUTES			PLASMA	R.B.C.	PLASMA SODIUM	PLASMA POTASSIUM	R.B.C. POTASSIUM	R.B.C. SODIUM
1.		36	48.7	0	33,700	480	134	4.1	80	14.8
2.	2	10	47.7	0	33,200	1370	133	4.2	83	15.2
3.	5	05	47.8	0	33,500	1800	135	3.8	81	14.2
4.	6	32	47.3	+	32,200	1870	135	3.6	82	12.5
5.	7	23	47.4	++	32,400	1900	132	3.6	81	12.5

I) and by removing most of the plasma and replacing it with isotonic NaCl or KCl (Experiment 19, Table I and Fig. 4, *C*).

In Experiment 14, the R.B.C. of patient A. G., who had a high R.B.C. sodium as well as apparently nonrapidly exchanging ("bound") red cell sodium, were tested after replacing most of her own plasma with plasma from a compatible donor with normal R.B.C. sodium. See Table I and Fig. 1 for the results.

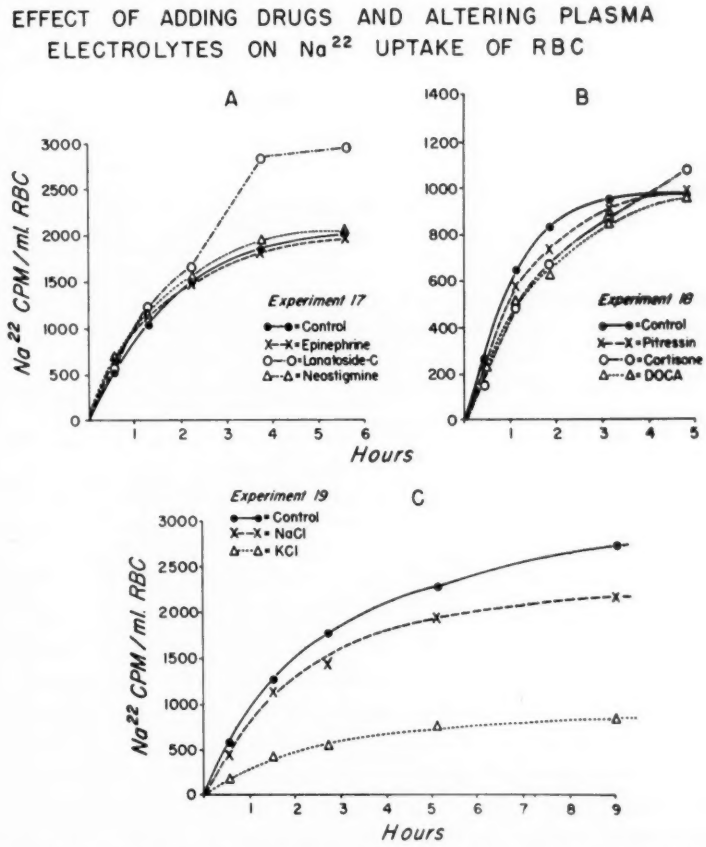

Fig. 4.—Rise in red cell Na^{22} with time in experiments in which drugs were added (*A* and *B*) and after aqueous solutions had been substituted for the plasma (*C*).

INTERPRETATION OF DATA

Relation of Total to Rapidly Exchanging Cell Sodium.—It is evident from Fig. 1 that there was considerable variation in the relationship of total to rapidly exchanging cell sodium. In twenty-two instances, the difference between these two values was less than 1.5 meq./L. of R.B.C. Because of the difficulties in chemical measurement of total red cell sodium, differences of this magnitude may be within the errors of the method. In the remaining

eight experiments, the rapidly exchanging sodium was found to vary from 1.9 to 5.9 meq./L. of R.B.C. lower than the total sodium. This suggests that sodium exists in red cells which does not exchange readily with the plasma sodium. The existence of "bound" sodium has been found previously in all subjects tested by C. W. Sheppard,[17] and this has been confirmed by Solomon.[18] These observers found the rapidly exchanging sodium to constitute characteristically less than half of the total red cell sodium. It is our opinion that the high total sodium values in our early experiments were due in part to contamination of the red cell solutions. This would contribute to the "bound" red cell sodium values. The five early experiments (Fig. 1) showing higher total sodium than rapidly exchanging sodium were done on five different individuals in each of whom subsequent measurements failed to confirm this finding. However, patient A. G. is an exception, since three determinations on two occasions showed from 2.4 to 5.9 meq./L. of "bound" red cell sodium. The validity of this finding is strengthened by the fact that the second two analyses were run in parallel with analyses on a subject who did not exhibit this phenomenon (Experiment 14, Table I).

Because of the small number of subjects involved, no general conclusions can be drawn from our data. However, the existence of "bound" sodium in the seven normal subjects we have studied is questionable. On the other hand, the presence of red cell sodium which does not readily exchange with the plasma sodium appears to have been confirmed in one patient with a relatively high total red cell sodium.

Exchange Rate.—From 2.9 to 4.4 meq. of sodium entered per liter of red cells per hour (mean value of 3.7), in the cells obtained from laboratory personnel and tested under standard conditions. This represents from 46 to 78 (mean 64) per cent of the exchanging sodium present in the R.B.C. These data agree well with those of Sheppard and of Solomon who reported values of 3.2 and 2.6 meq. Na/L. of R.B.C./hr. and 62 and 67 per cent, respectively.[17, 18]

Relation of Exchange Rate to Amount of Rapidly Exchanging Red Cell Sodium.—It would be of interest to know whether or not the amount of sodium entering and leaving the red cells was related quantitatively to the amount of rapidly exchanging sodium present. The data for the normal subjects are too few and closely grouped to establish any such relationship satisfactorily. In the two patients with high total red cell sodium concentrations, the exchange rate per hour in both was approximately half of the total rapidly exchanging sodium present, even though the absolute values of exchanging sodium were quite different, being 8.5 meq. in one instance and 13.3 meq. in the other. In Experiment 19 (Table I, Fig. 4, *C*), in which the medium bathing the red cells was changed by the addition of isotonic solutions of KCl and NaCl, the exchange rate per hour was from 36 to 39 per cent of the total exchanging R.B.C. sodium content. This was in spite of an absolute variation of exchange rate from 0.9 to 3.2 meq. Na/L. of R.B.C./hr., and of rapidly exchanging sodium content of from 2.5 to 8.3 meq. of Na/L. of R.B.C. Further experiments are necessary to confirm this relationship. In a study of the sodium exchange

rate of the erythrocytes of several different animals,[17] Sheppard found a suggestion of a relationship between the rate of erythrocyte sodium exchange and the amount of R.B.C. sodium present. Because of species differences the significance of these observations in evaluating the findings in man is uncertain.

Effect of the Temperature of Incubation on the Exchange Rate.—It has been shown that the speed of transfer of potassium into and out of red cells is very sensitive to changes in temperature.[16, 19] This has been considered to be evidence that a chemical reaction, such as glycolysis, is involved in control of the rate of exchange of potassium. Therefore, it was of interest to test the effect of lowering the incubation temperature on the rate of sodium transfer into and out of red cells. The transfer rates were compared at 38° C. and 28° C. in two subjects (Table I). The exchange rate at 38° C. on two occasions was 3.9 meq. Na/L. of R.B.C./hr. in subject A. F., or 69 and 57 per cent of the exchanging sodium. At 28° C. the value was 1.5 meq. Na/L. of R.B.C./hr., or 23 per cent of the exchanging sodium. The rates at 38° C. were 3.5 and 3.8 meq. Na/L. of R.B.C./hr., or 63 and 78 per cent of the exchanging sodium in subject R. M. On reducing the temperature to 28° C. the rate declined to 1.9 meq. Na/L. of R.B.C./hr., or 23 per cent of the exchanging sodium. Thus in subject A. F. the absolute and percentage transfer rates decreased an average of 62 and 63 per cent. The absolute rate fell 48 per cent, and the percentage rate 67 per cent in R. M. This change in transfer rate is of the same order observed for potassium transfer, and indicates the existence of a temperature sensitive chemical reaction involved in determining the rate of transport of sodium into and out of erythrocytes. It has previously been shown that cold-stored R.B.C. gain sodium and that warming the cells in the presence of glucose results in expulsion of sodium against a concentration gradient.[11, 12]

Influence of Potassium Level in the Plasma on Rate of Transfer of Sodium Into and Out of Red Cells.—Because of the known reciprocal relationship between the red cell sodium and potassium concentrations, we became interested in knowing whether or not the plasma potassium level had an influence on the sodium uptake rate of the red cells. In a single study (Experiment 10, Table I), raising the plasma potassium level by 10 meq./L. during the course of an experiment did not produce any change in the rate at which sodium entered the red cells. In Experiment 19, *C*, in which the potassium concentration of the medium in which the red cells were suspended was raised to 102 meq. K/L. by the addition of isotonic KCl solution, the sodium transfer rate of the cells was very low, amounting to 0.9 meq. Na/L. of R.B.C./hr. However, this finding was also associated with a low sodium concentration both in the medium (34 meq. Na/L.) and within the red cells, so that the influence of potassium per cent cannot be evaluated in this experiment. The influence of very low plasma potassium levels has not been tested. Harris and Maizels have reported that with potassium levels below 1 meq./L. active sodium transport in red cells is decreased.[20]

The Influence of Possible Plasma Factors on the Total and Rapidly Exchanging Red Cell Sodium Concentrations.—In Experiment 13, patient A. G. was shown to have red cells with a high total sodium content, and a significant part of this sodium was found to be in a nonrapidly exchanging ("bound") state. In order to determine whether these findings were associated with plasma factors which might be capable of producing the same state in normal cells in vitro, this patient's plasma was "crossed-over" with plasma from a compatible donor whose cells were normal. The cells of each subject were studied simultaneously in his own plasma and in the donor plasma. In both instances the red cells retained their respective original characteristics (Experiment 14, Table I and Fig. 1) despite suspension in plasma obtained from another sample of blood.

Influence of Drugs on Red Cell Sodium Metabolism.—Six drugs, selected because of known or suspected effects on electrolyte metabolism in cells, were tested for possible effects on red cell sodium metabolism. These were: epinephrine, neostigmine, lanatoside, pitressin cortisone, and DOCA.[21-23] They were added to the blood in amounts sufficient to produce concentrations of the same order as would be found in man if a full therapeutic dose were dissolved in the extracellular fluid. No striking effects were produced by these drugs (Fig. 1, Table I, Fig. 4, *A* and *B*). However, the significance of this finding is uncertain, because the drugs may not have been present in an active form after simple dilution with the plasma; and because significant changes in sodium metabolism might have been produced that were not detectable by the methods used. It should be emphasized that in an active and rapid exchange process between cells and extracellular fluid, a very small percentage change in the relationship of cellular intake to output can produce large net shifts of electrolyte in short periods of time.

GENERAL COMMENTS

These observations have importance in understanding sodium metabolism in a single type of human cell, i.e., the erythrocyte. It is possible that the generalities found for erythrocytes apply to the cells of other specialized tissues of the body. Obviously, the other cells must be studied directly.

In these experiments, the cells have been analyzed directly for radioactivity rather than by estimating the cellular content of tracer from that which disappears from the plasma. This removes the uncertainty caused by the presence of white cells and platelets in whole blood. The red cells were found to contain sodium in amounts smaller than those previously reported, and this sodium participated in a process of continuous exchange with the plasma sodium. This is a direct confirmation of the results of studies in intact man indicating an exchange of the sodium of the extracellular fluid with other stores of sodium presumably within living cells or in bone.[10, 24]

Although the sodium of red cells readily exchanges with that in the plasma, this exchange is not dependent solely on a passive process of diffusion across a membrane or similar barrier separating the cells and plasma, but is dependent at least in part on the rate of a chemical reaction taking place with-

in the system. This is supported by the fact that the transfer in and out of the cells is greatly influenced by temperature. It is interesting to note that approximately twice as many atoms of sodium enter and leave a red cell per unit time as do potassium atoms. This difference is not explained on the basis of differences in the concentration gradients of the elements involved, since the gradient for potassium is somewhat higher than that for sodium.

Evidence that sodium may exist in a "bound" state within the red cells is of considerable interest. The concept of inactive (osmotically) cellular base has been used to explain the apparently higher osmolarity of some cells as compared to the extracellular fluid.[25] Robinson[26] has found that the difference in apparent osmolarity between cells and extracellular fluid of tissue slices is maintained by the active metabolism of the cells. If energy were used by undisturbed living cells to "bind" base and make it osmotically inactive, the base might not mix or exchange with the other cellular contents. The magnitude of the sodium "bound" in the red cells is much too small, if it exists at all, to explain any significant osmotic effect. Nevertheless, the conclusive demonstration that the base could exist within any cell in a form not readily exchangeable, especially a nonnucleated cell, would be of general significance.

The in vitro system provided in studies of this sort gives an opportunity to measure the effects on cellular sodium metabolism of various drugs and chemicals of known clinical importance. In addition it is possible to remove from man, both healthy and diseased, a living cellular system for analysis in vitro, thus facilitating a study of possible abnormalities of cell electrolyte metabolism characteristic of disease.

SUMMARY

1. Human whole blood was maintained under simulated physiological conditions in vitro and the exchange of sodium between the plasma and red cells studied using Na^{22}.

2. In the majority of experiments, substantially all of the red cell sodium was found to exchange at a uniform and rapid rate with the plasma.

3. In one subject, there remained a portion of the red cell sodium which had not exchanged with the plasma in six hours.

4. A mean of 3.7 meq. of sodium entered a liter of red cells in one hour. This was 64 per cent of the rapidly exchanging sodium content of the cells.

5. Reducing the temperature at which the blood was incubated from 38° C. to 28° C. resulted in a more than 50 per cent decrease in the exchange rate.

6. The significance of these observations is discussed.

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A COMPARISON OF POTASSIUM^{42}, RUBIDIUM^{86}, AND CESIUM^{134} AS TRACERS OF POTASSIUM IN THE STUDY OF CATION METABOLISM OF HUMAN ERYTHROCYTES IN VITRO

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OUR interest in the mechanisms involved in the electrolyte metabolism of normal subjects and of patients with congestive heart failure prompted earlier investigations in which man as a whole was studied using long-lived isotopes of sodium and chlorine.[1, 2] The studies of potassium metabolism of human erythrocytes to be described in this report were undertaken in order to observe cellular electrolyte metabolism of man under controlled in vitro conditions where the difficulties inherent in the interpretation of experiments which involve the whole body could be avoided. In addition, the possible usefulness of the isotopes Rb^{86} ($t\frac{1}{2}$-19.5 days) and Cs^{134} ($t\frac{1}{2}$-2.3 years) as tracers of potassium was explored, since the short-lived isotope K^{42} ($t\frac{1}{2}$-12.4 hours) is not suitable for many types of investigation of potassium metabolism. The circulating red cells are being increasingly used in studies of clinical electrolyte physiology, since they are easily "biopsied" by venipuncture[3-6] and are better suited than most cells for in vitro experiments. Human erythrocytes have the high potassium and low sodium concentrations found in the cytoplasm of most types of body cells, and the maintenance of these concentrations is associated with active enzymatic and chemical processes, including glycolysis.[7, 8] The sodium and potassium metabolism of erythrocytes (R.B.C.) has been extensively studied, and within recent years the availability of radioactive traces has made it possible to demonstrate conclusively that there is a continuous active transfer of both sodium and potassium into and out of the red cells, even under conditions where the intracellular and extracellular concentrations of these elements are constant.[9-11] Rubidium and cesium are the heavier members of the same group of elements as sodium and potassium, and resemble potassium in many of their biological properties. A similarity in the metabolism of potassium, rubidium, and cesium by red cells is therefore to be expected. If the similarity were great, the slow physical decay of Rb^{86} ($t\frac{1}{2}$-19.5 days) and Cs^{134} ($t\frac{1}{2}$-2.3 years) might make these isotopes useful in long-term biological experiments unapproachable with the short-lived isotope K^{42} ($t\frac{1}{2}$-12.4 hours) which is the only readily available radioactive isotope of potassium.

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The general plan of study in the experiments to be reported was as follows: human whole blood was maintained under simulated physiological conditions in vitro, and measurements were made of the rate with which added K^{42}, Rb^{86}, and Cs^{134} passed from the plasma into the red cells. In order to determine the extent to which Rb^{86} and Cs^{134} traced potassium in this one type of cellular system, the radioactivity of Rb^{86} and Cs^{134} was treated as if it were due to radioactive potassium, just as when K^{42} was used. The ratios Rb^{86} counts per milliequivalent K^{39} and Cs^{134} counts per milliequivalent K^{39} were used as if they represented specific activities of potassium, and calculations of the apparent potassium exchange rate between R.B.C. and plasma were made using the methods of Sheppard.[9] This resulted in values which could be compared with those obtained with K^{42} in our experiments as well as with reported values of others.[9, 10] Thus the possible usefulness of Rb^{86} and Cs^{134} as tracers for potassium was tested. It is important to realize that calculations of potassium exchange rate based on the premise that Rb^{86} and Cs^{134} label potassium do not actually represent measurements of potassium exchange rate, nor are they rubidium or cesium exchange rates. For this reason, the symbols ER_{Rb}, and ER_{Cs} are used in this report to represent values that do not necessarily reflect potassium exchange rates, but rather the result of arbitrary calculations made to determine the relationship between the three elements used as tracers. That is, ER_{Rb} and ER_{Cs} are the result of calculations made with the premise that Rb^{86} and Cs^{134} trace potassium, and are expressed as amount of K^{39} apparently transferred from red cells to plasma per unit time. Similarly ER_K is the result of the same calculations when K^{42} was used to trace K^{39}. In addition to measuring the ER_K, ER_{Rb}, and ER_{Cs}, the studies included: (1) measurements of the eventual quantitative distribution of each element between R.B.C. and plasma after it was originally added to the plasma, (2) observations of the influence of changes in temperature on the ER_{Rb} and ER_{Cs}, and (3) evaluation of the effect of changes in the level of concentration of potassium and rubidium in the plasma on the uptake of each element by the red cells.

MATERIAL AND METHODS

Blood Studied.—The blood studied was obtained from laboratory personnel, and in two instances from patients hospitalized with congestive heart failure. Venous blood was drawn with stasis through an 18-gauge needle from the median basilic vein and heparinized by the addition of 10 mg. of heparin in 1 ml. of 0.15M NaCl solution per 50 ml. of blood. Both heparin without preservative and with ½ mg. of chlorobutanol per milligram of heparin were used. No differences in the results associated with the presence of preservative were observed.

Method of Incubation.—After obtaining the blood, a portion was used for initial chemical determinations and to obtain plasma for "tagging" with the radioelement. The remainder was transferred to paraffin-coated flasks for incubation by one of two alternate methods.

The *first* was described by C. W. Sheppard.[9] By this method a series of individual Erlenmeyer flasks were used whose flat bottoms were ridged to separate the cells and tagging plasma during an initial period of equilibration of the blood to the environment of the experiment, and whose tops allowed the atmosphere above the contained blood to be controlled by a continuous flow of pre-mixed gases. A number of these flasks, one for each aliquot measured, were placed in a rack and submerged in a water bath where they were maintained at 38.0 ±

.05° C. At the instant selected for the mixing of blood and plasma, thirty to forty-five minutes after placing the blood in the flasks, the rack was begun rotating through a 90 degree arc ten times a minute. Since the ridge in the bottom of the flasks rested at a 45 degree angle to the axis of rotation, mixing was achieved by the spilling of the contents first over one and then over the other end of the ridge. A mixture of 8 per cent oxygen, 86 per cent nitrogen, and 6 per cent carbon dioxide, saturated with water at 38° C., was flushed through the flasks rapidly at first and then during the course of the experiment at approximately five liters per hour. This technique assures accurate timing of the moment the isotope and blood are mixed, and also supplies a gentle method for maintaining the blood in a uniformly mixed state. However, it is inconvenient because of the number of flasks employed, and because differences in the amount of isotope per unit volume of blood are inevitably present from flask to flask. This necessitates a correction based on the measured radioactivity per milliliter of whole blood for each flask.

A *second* method was introduced to avoid the difficulties offered by Sheppard's method and to simplify the procedure. A single 200 ml. round-bottomed paraffin-coated flask, fitted with a top allowing gas flow and sample removal, was used. Fifty milliliters or more of blood could be incubated in one bottle and an aliquot removed at any desired time for study. This allowed ten specimens to be taken without correction for variations in radioactivity among aliquots. Mixing was obtained by a mechanical rack which rotated the flasks through a 30 degree arc, at a radius of 7 cm., seventy times a minute. There were certain disadvantages. These were: slower and somewhat less precisely timed mixing of all the blood with the isotope; slow equilibration of the larger volume of blood with the gas atmosphere; and the necessity for opening the flask to the air briefly during sampling. These did not prove important, for no differences in the results with the two methods were found. Raker and associates[10] have found that no effect was produced on R.B.C. potassium exchange by varying the atmospheric carbon dioxide from 2.5 to 20 per cent and the oxygen from zero to 95 per cent, so that minor changes in gas tensions would not be expected to have measurable influences. With either of these systems of incubation, blood could be maintained for four to twenty-four hours without visible hemolysis, rise in the hematocrit, or rise in plasma potassium.

Adding the Tracer.—One to two milliliters of plasma containing the desired amount of radioelements was mixed with the whole blood at zero time. This plasma comprised less than 5 per cent of the total volume of blood, and contained the isotope together with sufficient glucose to raise the initial blood glucose by 250 mg. per cent.

Radioelements Used.—K^{42}, Rb^{86}, and Cs^{134} were obtained as carbonates from the Isotopes Division of the Oak Ridge National Laboratories, neutralized with HCl, and brought to the desired concentration of the radioelement. Physical decay and aluminum absorption studies showed that the solution of K^{42} contained less than 0.01 per cent of a radioactive substance indistinguishable from Rb^{86}. We could not definitely exclude contamination in the laboratory as the source of this element, but this was unlikely. All shipments of Rb^{86} tested have shown the presence of larger amounts of a longer-lived radioactive element which had a physical half-life and aluminum absorption curve indistinguishable from Cs^{134}. It was handled by red cells in the same manner as Cs^{134} (Experiment C-3, Table III). The presence of this material was not due to laboratory contamination. This contaminant accounted for approximately 0.5 per cent of the radiation of the specimen when first received from Oak Ridge. As time elapsed, the percentage of radiation due to the contaminant rapidly increased in a manner expected theoretically from a mixture of Rb^{86} and Cs^{134}. In addition, the possibility of biological separation of this element by the system being studied adds to the importance of its proper consideration. In those experiments done with some of the material allowed to stand around the laboratory for a few weeks, recounting of specimens after physical decay of the Rb^{86} indicated the need for a significant correction in one experiment (Experiment B-6, Table III).

In all experiments samples were collected for radioactivity measurements at appropriate intervals after adding the isotope. Simultaneous determination of hematocrit and potassium concentration of plasma and red cells were obtained.

Hematocrit.—Hard glass tubing of outer diameter 4 mm. and inner diameter 2.5 mm. was sealed at one end with a flame to form hematocrit tubes containing an 80 mm. column of blood. Centrifugation was carried out in an angle centrifuge at 20,000 RCF for ten minutes, at which time maximal detectable packing had occurred. After angle centrifugation, the top of the packed cell column was flattened by ten minutes of centrifugation at right angles in a field of 1,750 RCF, and readings obtained using a horizontal millimeter scale under a microscope at low magnification. This method was reproducible within 0.7 per cent, and a correction of the resulting values was applied on the premise that red cells packed under these conditions contain 1.9 per cent plasma. The nature of this correction factor, the technique of red cell separation, and the chemical methods employed are discussed in detail elsewhere.[12] In some instances the hematocrit, together with the plasma and whole blood values, was used to calculate the red cell potassium or radioactivity. Alternatively, the red cells were collected and studied directly.

Red-Cell Separation.—In order to obtain red cells with a minimal and constant amount of plasma contamination, whole blood was centrifuged in plastic tubes under the same conditions as the initial centrifugation for the hematocrit. A special clamp[12] was then placed on the tube at a point 2 mm. below the buffy coat, and the contents trapped below the clamp separated from the supernatant which contained over 95 per cent of the white cells. To permit pipetting, the cells were hemolyzed by freezing. Like the packed cells of the hematocrit, the red cells were considered to contain 1.9 per cent plasma, and the appropriate correction was made.

Chemical Determinations.—Potassium was determined with a modified Beckman model DU flame photometer making suitable corrections for the presence of sodium in the plasma and whole blood. A 67.7 fold dilution (0.15 ml. into 10 ml.) of hemolyzed red cells produced values in good agreement with solutions in which the cells had been digested with nitric acid, so that the simple dilution procedure was used.

Determination of Radioactivity of Samples.—Samples were prepared on filter paper discs by methods previously described[13] and counted with a thin mica end window Geiger-Müller counter. A detergent solution (1.7 per cent "Hemosol") was used to aid in giving even distribution of the red cells on the filter paper discs. The whole blood was hemolyzed by freezing before pipetting. When equal amounts of Rb^{86} were added to equal volumes of red cells and plasma and slides made according to the technique used, no consistent differences in apparent radioactivity were noted between the cells and plasma. In experiments where K^{42} and Rb^{86} were both present, preparations were counted immediately and again after the K^{42} had decayed to a negligible amount. By suitable corrections for physical decay, the amounts of each isotope present at the time of the experiment were determined.

Calculations.—Calculations of the rates of exchange of substances betwen stable compartments to which tracers have been added have been discussed extensively in the literature.[9, 10, 14] Our calculations have been made using Sheppard's formulations for the two-compartment system.[9] The exchange rate is defined as the amount of potassium entering or leaving either the plasma or the red cells per unit time. In the final form it is given by:

$$\rho = \frac{0.69 S_1 S_2}{t\frac{1}{2}(S_1 + S_2)}$$

where

ρ = the exchange rate per unit volume of whole blood,
S_1 = the total plasma potassium per unit volume of whole blood,
S_2 = the total red cell potassium per unit volume of whole blood, and
$T\frac{1}{2}$ = the time for equalization of specific activity in both compartments to proceed half way.

For purposes of comparison, ρ was expressed as per cent of the cellular potassium and as the amount of potassium transferred per unit volume of cells per unit time. $t\frac{1}{2}$ was obtained in a variety of ways. The difference between the specific activity of either compartment and the whole blood specific activity when plotted against time semilogarithmically

TABLE I. DATA ON EXPERIMENT A-1 IN WHICH K^{42} AND Rb^{86} WERE USED SIMULTANEOUSLY AND CALCULATIONS OF THE EXCHANGE RATE OF POTASSIUM BETWEEN THE R.B.C. AND PLASMA MADE OF THE PREMISE THAT BOTH ISOTOPES LABELED POTASSIUM. BLOOD WAS OBTAINED FROM A NORMAL SUBJECT AND INCUBATED AT 38° C. IN A SINGLE FLASK. THE OBSERVED R.B.C. POTASSIUM WAS 88 MEQ./L., AND THE CALCULATED PLASMA RADIOACTIVITIES AT ZERO TIME WERE: K^{42}, 6,380 CTS./MIN./ML.; Rb^{86}, 4,440 CTS./MIN./ML.

SPECIMEN NUMBER	TIME HR.	TIME MIN.	HEMATOCRIT	K^{42} (CTS./MIN./ML.) PLASMA	K^{42} (CTS./MIN./ML.) WHOLE BLOOD	Rb^{86} (CTS./MIN./ML.) PLASMA	Rb^{86} (CTS./MIN./ML.) WHOLE BLOOD	PLASMA K MEQ./L.	HEMOLYSIS
1		46	50.5	3,740	3,200	2,600	2,100	3.5	0
2	1	15	51.5	2,970	3,150	2,080	2,160	3.4	0
3	1	47	51.0	2,420	2,950	1,590	2,400	3.4	0
4	2	17		1,910	3,220	1,220	2,120	3.4	0
5	4	18	50.6	840	3,150	550	2,190	3.0	0
6	6	23	50.2	550	3,100	260	2,210	2.9	0
7	10	02	51.1	300	3,150	190	2,120	2.9	0
8	13	10	50.6	250	3,080	180	2,170	3.0	0

resulted in a straight line from which the half time was determined. Alternatively, the same information was derived from a similar plot of the difference between specific activities in the two compartments. These calculations apply only to a closed system of two uniformly mixed compartments in which exchange rate is constant and there is no accumulation of the nontracer in one compartment or the other. It is clear that whole blood actually consists of one large plasma compartment which communicates separately with each red cell, white cell, and platelet. Sheppard found the buffy coat of dog's blood to contain a significant amount of relatively rapidly exchanging potassium.[15] However, in human blood the error in red cell studies due to admixture of leukocytes is smaller than in dogs.[9, 10] The plot from which the $T\frac{1}{2}$ was obtained was employed as a partial index of the reliability of the calculated rate of exchange. This is true because deviations from a straight line indicated technical error, inhomogeneity of rates of exchange, or change in the rate of exchange. There has been a tendency in our experiments and in those of others for a fall in the plasma potassium to occur during the incubation of blood. This has been shown to be associated with glycolysis by the cells and a net uptake of potassium by them.[7] When marked, this change in the compartment content decreases the usefulness of the calculations. As discussed above, the ρ values obtained from these calculations are referred to in this report as ER_K, ER_{Rb}, or ER_{Cs}, depending on the isotope used.

RESULTS

The data obtained allowed the comparison of potassium, rubidium, and cesium in four aspects of their metabolism by R.B.C. There were: *first,* the partition of each element between the R.B.C. and plasma after final stable dis-

TABLE II. DATA OF EXPERIMENT C-1 IN WHICH THE SPEED WITH WHICH Cs^{134} ENTERED THE R.B.C. FROM THE PLASMA WAS MEASURED. ON THE PREMISE THAT Cs^{134} WAS LABELING POTASSIUM, CALCULATIONS OF THE POTASSIUM EXCHANGE RATE WERE MADE FOR COMPARISON WITH RATES OBTAINED USING K^{42} AND Rb^{86} AS THE TRACERS (TABLE III). THE BLOOD OF A NORMAL SUBJECT WAS INCUBATED AT 38° C. IN A SINGLE FLASK. THE OBSERVED R.B.C. POTASSIUM WAS 83 MEQ./L., AND THE CALCULATED PLASMA RADIOACTIVITY AT ZERO TIME 16,040 CTS./MIN./ML.

SPECIMEN NUMBER	TIME HR.	TIME MIN.	HEMATOCRIT	Cs^{134} (CTS./MIN./ML.) PLASMA	Cs^{134} (CTS./MIN./ML.) RED CELLS	PLASMA POTASSIUM MEQ./L.	HEMOLYSIS
1		13	43.5	16,070	310	3.7	0
2		57	43.5	15,050	1,390	3.6	0
3	3	15	43.2	12,670	4,000	3.3	±
4	6		43.8	10,440	6,560	3.2	+
5	11	44	43.8	7,740	9,830	3.6	++

tribution of the tracer had been nearly reached; *second,* the speed with which final levels were approached, i.e., the ER_K, ER_{Rb}, and ER_{Cs}; *third,* the effects of temperature change on the ER values; and *fourth,* the effect of changes in the plasma potassium and rubidium levels on the rate of disappearance of the isotopes used from the plasma.

The Partition of K^{42}, Rb^{86}, and Cs^{134} Between R.B.C. and Plasma.—The time course of the distribution of K^{42}, Rb^{86}, and Cs^{134} between R.B.C. and plasma is compared in Fig. 1, the fall in plasma "specific activity" (K^{42}/K^{39}, Rb^{86}/K^{39}, Cs^{134}/K^{39}) with time being shown. The specific activities are given as per cent of the whole blood specific activity, so that if the plasma specific activity falls to that of the whole blood, the relative intracellular and extracellular partitioning of the isotope in question is similar to that of potassium. The curves for

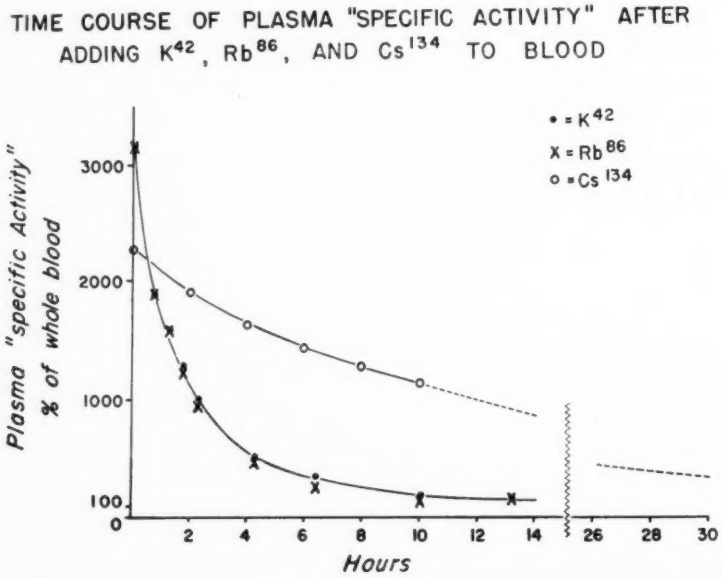

Fig. 1.—The time course of plasma "specific activity" (K^{42}/K^{39}, Rb^{86}/K^{39}, Cs^{134}/K^{39}) after adding radioactive isotopes to the whole blood. When the plasma "specific activity" falls to that of the whole blood, the isotope has become concentrated in the R.B.C. to the same extent as potassium (K^{39}).

K^{42} and Rb^{86} specific activity were derived from Experiment A-1 (Table I) in which both isotopes were observed simultaneously. It can be seen that the plasma specific activity in both instances approached the whole blood value asymptotically. In the same way, direct measurement showed the red blood cell specific activities to rise and approach essentially the same value. Because of its slower rate of disappearance from the plasma, cesium could not be followed until it approached the final concentration closely. Instead, after Experment C-1 (Table II) had shown that Cs^{134} was definitely concentrated within red cells, the data were analyzed to obtain a $t_{1/2}$ time and the plasma and red cell curves were then extended by extrapolation. These curves indicated that the plasma and red cell specific activities approached the whole blood value

asymptotically, and, therefore, that all three ions had distributed themselves similarly to the naturally occurring potassium. This was further shown to be true for Cs^{134} in an experiment in which the hematocrit was raised to 67.6 by removal of plasma (Experiment C-4, Table III). This accelerated the speed at which the final concentration was approached, so that after ten hours the ratio

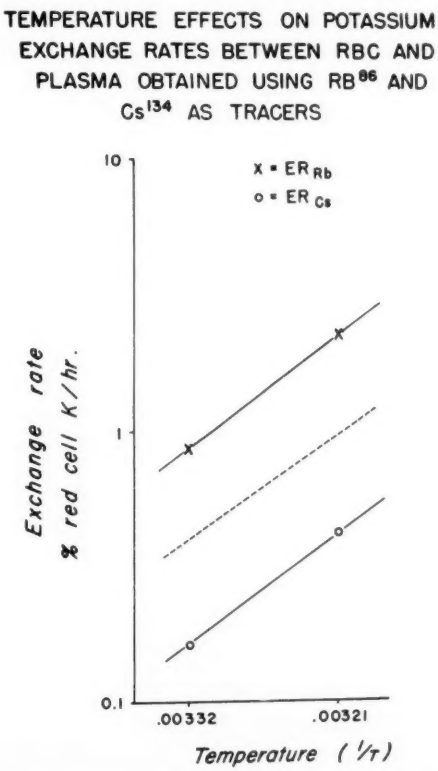

Fig. 2.—The extent of the slowing of ER_{Rb} and ER_{Cs} by lowering the temperature of the blood from 38° C. to 28° C. is shown. ER_{Rb} and ER_{Cs} are the results of calculations of potassium exchange rate between R.B.C. and plasma based on the premise that Rb^{86} and Cs^{134} traced potassium. The exchange rate is expressed as per cent of the red cell potassium entering or leaving per hour, and temperature as the reciprocal of the absolute temperature. The dotted line illustrates the slope that would be found if the Q_{10} were 2.35, the value obtained by Sheppard who used K^{42} to trace potassium. This line is nearly parallel to those obtained by experiments with Rb^{86} and Cs^{134} as tracers.

of plasma to R.B.C. specific activity was ten to one, and after thirty hours, three to two. This represented only a twofold concentration of Cs^{134} in the R.B.C., since in the latter hours of the experiment potassium was lost from the red cells into the plasma.

The Speed with Which K^{42}, Rb^{86}, and Cs^{134} Were Distributed Between R.B.C. and Plasma.—The ER values of all experiments are summarized in Table III. K^{42} was used simultaneously twice with Rb^{86}, and there was no detectable difference between ER_K and ER_{Rb} in either case. ER_{Cs} was much less than ER_K and ER_{Rb}, and in the best experiment was 0.3 meq. K/L. R.B.C./hr. compared with the mean ER_{Rb} value of 2.0 meq. K/L. R.B.C./hr.

The Effect of Changes in Temperature of Incubation on the Speed of Distribution of Rb^{86} and Cs^{134}.—The in vitro transfer of K^{42} between plasma and erythrocytes has been shown by others to be very sensitive to changes in the temperature at which the blood is incubated,[9, 10] so that between 15° C. and 44° C., each ten degree rise in temperature results in a more than twofold increase in the transfer rate (Q_{10} 2.2-2.35). In order to determine if the effect of temperature on ER_{Rb} and ER_{Cs} was of the same order of magnitude as for ER_K, experiments were done at 28° C. and 38° C. The results (Fig. 2) show that the depression of ER_{Rb} and ER_{Cs} by a ten degree decline in temperature was approximately the same as that reported for K^{42} (ER_K).

TABLE III. SUMMARY OF ALL EXPERIMENTS IN WHICH CALCULATIONS OF THE EXCHANGE RATE OF POTASSIUM BETWEEN R.B.C. AND PLASMA WERE MADE. THESE CALCULATIONS WERE BASED ON THE PREMISE THAT Rb^{86} AND Cs^{134} LABELED POTASSIUM, JUST AS DOES K^{42}. IN THE TEXT THESE VALUES ARE REPRESENTED BY THE SYMBOLS ER_{Rb}, ER_{Cs}, AND ER_K. R.B.C. POTASSIUM WAS EITHER CALCULATED FROM THE WHOLE BLOOD AND PLASMA POTASSIUM, USING THE HEMATOCRIT, OR MEASURED DIRECTLY ON SEPARATED R.B.C. (OBSERVED R.B.C. POTASSIUM)

EXPERIMENT	ISOTOPE USED	DONOR	NUMBER OF FLASKS	RED CELL POTASSIUM MEQ./L. OF R.B.C.	ER VALUE AS PER CENT OF R.B.C. K TRANSFERRED/HR.	ER VALUE AS MEQ. K TRANSFERRED PER L. OF R.B.C./HR.
A-1	K^{42} and Rb^{86}	Normal	Single	82 observed	1.8	1.5
					2.0	1.6
A-2	K^{42} and Rb^{86}	Normal	Single	88 observed	2.8	2.4
					2.8	2.4
B-1	Rb^{86}	Normal	Multiple	103 calculated	2.3	2.0
B-2	Rb^{86}	Patient	Multiple	109 calculated	2.2	2.0
B-3	Rb^{86}	Normal	Multiple	96 calculated	2.0	1.9
B-4	Rb^{86}	Patient	Multiple	110 calculated	1.9	2.1
B-5	Rb^{86}	Normal	Multiple	99 calculated	2.4	2.4
B-6	Rb^{86}	Normal	Single	97 observed	2.2	2.1
B-7	Rb^{86}	Normal	Single	82 observed	2.1	1.7
C-1	Cs^{134}	Normal	Single	83 observed	0.4	0.3
C-2	Cs^{134}	Normal	Multiple	89 calculated	0.6	0.5
C-3	Cs^{134}?	Normal	Single	97 observed	0.4	0.4
C-4	Cs^{134}	Normal	Single	89 observed	0.3	0.3
Mean Values					K^{42} 2.3	2.0
					Rb^{86} 2.1	2.0
					Cs^{134} 0.4	0.4

The Effect of Plasma Potassium and Rubidium Concentrations on the Rate of Disappearance of Isotopes from the Plasma.—It is known that ER_K is independent of the plasma potassium level between 2 meq./L. and 74 meq./L.[10, 11] Since the absolute amount of potassium leaving the plasma compartment per unit time in a given sample of blood is therefore reasonably constant, doubling the plasma potassium concentration would reduce the rate of fall of plasma radioactivity (K^{42}) by one-half. It was of interest, therefore, to learn the influence of plasma potassium level upon the rate of fall of plasma Rb^{86} and Cs^{134}. It was found that the rate of fall of plasma Rb^{86} was reduced by a rise in plasma potassium in a fashion similar to that expected if K^{42} had been employed as the tracer. A similar reduction was found with Cs^{134}. In other experiments the influence of plasma rubidium concentration on the behavior of Rb^{86} was ob-

served. In the several measurements of ER_{Rb}, the amount of nontracer rubidium in the plasma was estimated to have been raised by approximately 3 to 8 mg./L. when Rb^{86} was added. This was from three to eight times the reported naturally occurring level of plasma rubidium.[16] If rubidium were independent of potassium, a variable rise in the plasma rubidium might result in wide variations in the fall of plasma radioactivity and in the ER_{Rb} values. No such range of values was observed. In a single experiment in which K^{42} and Rb^{86} were employed simultaneously, after the experiment was already under way tracer-free rubidium was added in an amount that was insignificant compared to the potassium present, but several times the amount of rubidium already present. This resulted in no detectable change in the disappearance rate of either K^{42} or Rb^{86} from the plasma. However, when nontracer rubidium was added in an amount comparable to the potassium level, slowing of the disappearance of both K^{42} and Rb^{86} occurred.

DISCUSSION

These experiments show a remarkable qualitative similarity of the metabolism of potassium, rubidium, and cesium by red cells in vitro. The data obtained in these experiments indicate that both rubidium and cesium are concentrated within red cells to approximately the same extent as is potassium. This is contradictory to the results of Bertrand,[16, 17] who found by spectrographic analysis that there was only three to four times as much rubidium in the red cells as plasma. One source of difference in the results may be that the comparatively large amounts of rubidium added by us disturbed normal rubidium distribution, so as to make it resemble that of potassium. In the case of cesium, naturally present material has not been reported in the plasma or erythrocytes, and the amount of cesium that was added in these experiments is certainly much more than that normally present.

The mean exchange rate obtained in these experiments with Rb^{86} (ER_{Rb}) shows fair agreement with the reported results of others in which K^{42} was used (ER_K). Sheppard and Martin[9] have found the rate of exchange of potassium between R.B.C. and plasma to be 1.8 per cent of the cellular potassium per hour and Raker and associates[10] 1.6 per cent, while Solomon[18] has obtained a value of 1.87 per cent. The mean value using Rb^{86} as the tracer was 2.1 per cent. In the case of the measurements made with Rb^{86}, the comparatively small difference from the reported results with K^{42} may be due to differences in technique or to variation among donors, as well as to the tracer used.

The results of workers using K^{42} showed a Q_{10} of 2.2 to 2.35 for the potassium exchange by red cells. This has supported the conclusion that the transfer of potassium between red cells and plasma is dependent on a chemical reaction, and not solely on diffusion. The fact that the influence of temperature on the ER_{Rb} and ER_{Cs} compares well with that on ER_K indicates similarity in the actual process of transfer of these ions into the red cells, including active metabolic processes.

Solomon has previously reported that adding rubidium to the plasma decreased the uptake rate of K^{42} by red cells, and the data of this report show

in addition that the uptake of Rb^{86} and Cs^{134} are similarly reduced by increases in plasma potassium. These results collectively suggest that the plasma potassium, rubidium, and cesium are all part of a pool of ions which enter into apparently similar metabolic processes of red cells. The studies suggested only qualitative differences for these three ions. The uptake process for each ion is apparently qualitatively similar, but the ER_{Cs} is only one-fifth of the ER_K and ER_{Rb}. This may be related to the atomic size or weight of this element. In any case, it appears that the eventual quantitative distribution of Cs^{134} in blood is similar to that of Rb^{86} and K^{42}. The results of spectrographic examination on other biological material show that rubidium is widely distributed in nature, having been found in plants and in the organs of man and other animals,[19, 20] where its presence parallels that of potassium. Cesium, on the other hand, has not been found in biological material with one exception. It has been demonstrated in the retinae of oxen, pigs, and sheep where, because of its photoelectric properties, it may perform a special function in vision.[21] The degree to which rubidium or cesium can replace potassium in maintaining the normal function of several living systems has been studied. In general, substitution is not perfect, but it tends to be better with rubidium than with cesium. This has been true in isolated nerve and muscle preparations,[22-25] in hearts studied in vitro,[26] and in the in vitro formation of acetylcholine by brain tissue.[27] In potassium deficient rats rubidium and cesium enter the muscles in high concentrations and prolong life.[28, 29] Rubidium will prevent the anatomic lesions of potassium deficiency, but cesium will not, and in either case there are signs of central nervous system irritability with convulsions and death.[28, 29]

Since these differences between the biological properties of potassium, rubidium, and cesium are known to exist, in any instance where Rb^{86} is to be used to "trace" potassium it will be necessary to show identical behavior of isotopic potassium and rubidium. For this reason the usefulness of Rb^{86} may be greatest in pilot experiments. Absolute reliance on the metabolic similarity of rubidium and potassium is not justified, even in erythrocytes. On the other hand, the use of Rb^{86} in experiments requiring long periods of time may give useful information of a qualitative nature concerning potassium metabolism. This situation is analogous to the use of bromide to trace chloride, or of tritium or deuterium to trace hydrogen.

SUMMARY

1. The usefulness of Rb^{86} and Cs^{134} as tracers of potassium in the in vitro study of erythrocyte electrolyte metabolism has been explored using K^{42} as the standard of reference.

2. The eventual quantitative partition of these isotopes between the erythrocytes and plasma, after they were added to the plasma, was found to be nearly the same.

3. The calculated exchange rates of potassium between erythrocytes and plasma using K^{42} or Rb^{86} as the tracer were not detectably different by the methods used.

4. The mean potassium exchange rate, as measured with Rb^{86} as the tracer, was 2.1 per cent of the red cell potassium per hour.

5. The exchange measured with Cs^{134} as the tracer was less than one-fifth as great.

6. The effect of changes in the temperature of incubation and of raising the plasma potassium concentration was the same for each isotope.

7. The results suggest that Rb^{86}, with its half-life of 19.5 days, may be useful under some circumstances as a tracer for potassium, at least from a qualitative point of view. Pertinent data should be validated with K^{42} wherever possible.

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OBSERVATIONS ON THE ETIOLOGIC RELATIONSHIP OF ACHYLIA GASTRICA TO PERNICIOUS ANEMIA

XV. Hematopoietic Effects of Simultaneous Intravenous and of Simultaneous or Serial Oral Administration of Intrinsic Factor and Vitamin B_{12}

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PREVIOUS observations have shown potentiation of the hematopoietic activity of sources of vitamin B_{12} such as beef muscle, liver or liver extract, or of vitamin B_{12} itself,[1, 2] upon simultaneous oral administration with normal human gastric juice to patients with pernicious anemia. Despite earlier uncertainty,[3] alcoholic extracts of beef muscle have not been found to be hematopoietically more active than is vitamin B_{12} in microbially equivalent amounts when orally administered with gastric juice, or when injected parenterally without it.[4] However, vitamin B_{12} is invariably more potent upon injection than when given orally in equal quantity with gastric juice[1]; and it has been shown to cause local maturation of megaloblasts upon direct instillation into the marrow cavity.[5]

The simplest explanation of these facts is that the so-called gastric (intrinsic) factor has no other function than to promote the absorption of vitamin B_{12} from the alimentary tract. This supposition has received substantial support from recent measurements of decreased fecal output of radioactivity following the simultaneous oral administration of 0.5 μg amounts of radioactive cobalt-containing vitamin B_{12} and intrinsic factor.[6] Indeed, the increased amounts of vitamin B_{12} that have thus by inference been absorbed are sufficient to account for the moderate hematopoietic effects noted in similar observations[1, 2, 7] with only slightly larger amounts of vitamin B_{12}. However, it is possible that in addition to increasing the absorption of vitamin B_{12}, the gastric (intrinsic) factor possesses the property of rendering vitamin B_{12} hematopoietically more active by forming some type of combination with gastric juice. Gastric juice has been noted to possess a "binding" effect upon vitamin B_{12} which with stoichiometric proportionality renders the vitamin unavailable for microbial growth.[8] Moreover, it has been claimed that gastric juice must be added to vitamin B_{12} in order for the latter to "ripen" the megaloblasts in cultures of pernicious anemia bone marrow in vitro.[9]

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The clinical observations reported below attempt to provide answers to three questions concerning the nature of the essential physiologic function of intrinsic factor: (1) Is the hematopoietic activity of vitamin B_{12} increased upon simultaneous intravenous injection with intrinsic factor? (2) Does a stoichiometric relation exist between vitamin B_{12} and intrinsic factor with respect to the potentiation of vitamin B_{12} by intrinsic factor upon simultaneous oral administration? (3) Does the order of the serial oral administration of vitamin B_{12} and of intrinsic factor influence the hematopoietic effect of the vitamin B_{12}?

METHODS

All patients studied had pernicious anemia either in relapse or without previous treatment. The diagnosis was established by the characteristic blood and bone marrow morphology and by the presence of histamine-fast gastric achlorhydria. In one patient the entire stomach had been removed by previous surgery. The diagnosis was confirmed in each patient by one or more reticulocyte responses to vitamin B_{12} and eventually by return to normal blood values as a result of further therapy. During the observations the basal diet of the patients contained no meat, fish, or eggs, and little milk. When preparations containing gastric juice were administered by mouth, the last meal of the day (consisting of tea, toast, and jelly) was given at 4 P.M. Unless otherwise indicated, all test substances were administered orally at 8 P.M. No food was allowed until breakfast the following morning.

Hemoglobin, hematocrit, and red cell determinations were made approximately every third day on venous blood samples, using balanced oxalate as anticoagulant. Daily reticulocyte counts were made on capillary blood samples, employing coverslips previously coated with a film of dried brilliant cresyl blue and subsequently counterstained with Wright stain. The method of serial reticulocyte responses[10] was employed for the evaluation of the relative erythropoietic potencies of substances given in uniform daily amounts during each of two or more consecutive periods of ten or more days each. When a second reticulocyte peak appears, irrespective of its magnitude relative to the first, the material causing the second peak is considered to be the more active. This principle applies to consecutive periods of observation yielding more than two reticulocyte peaks.

Gastric juice was collected usually daily from normal fasting individuals known to secrete intrinsic factor. The donor's stomach was first rinsed with sterile distilled water, then the gastric juice secreted for about an hour after one or two subcutaneous injections of 0.5 mg. of histamine phosphate was collected in a sterile flask. After standing in the icebox for an hour or more in order to allow gross mucus to settle, the supernatant material was passed through a coarse sintered glass filter and brought to neutrality with 20 per cent sodium hydroxide solution. Thereafter it was usually put through a Berkefeld V filter and stored frozen at −32° C. Ordinarily a pool totaling 400 ml. was collected in advance of each observation.

Vitamin B_{12} solution* was obtained in sealed glass ampules containing 15 μg per milliliter. One milliliter of this solution was carefully diluted with sterile isotonic saline to give the final concentration desired. The resulting dilute vitamin B_{12} solution was kept in a rubber-capped vial, which was stored in the icebox at 4° C. and was used as the source of vitamin B_{12} throughout each observation. Each dose, always made equal to 1 ml., was accurately measured when removed with a sterile tuberculin syringe and needle.

OBSERVATIONS

Is the hematopoietic activity of vitamin B_{12} increased upon simultaneous intravenous injection with intrinsic factor?—It has been shown that the intravenous administration of as much as 190 ml. of sterile neutral gastric juice over

*Kindly supplied by Dr. Augustus Gibson of Merck & Co., Inc., Rahway, N. J.

a period of six days has no deleterious or erythropoietic effects in pernicious anemia.[11] This indicates, as does microbial analysis in our hands, a lack of significant amounts of vitamin B_{12} in Berkefeld-filtered normal human gastric juice. The discovery that the activity of as little as 5 μg[2, 7] (or, as will be shown below, of 1 μg) of vitamin B_{12} in pernicious anemia can be enhanced by as little as 10 ml. of sterile Berkefeld-filtered gastric juice when given simultaneously daily by mouth, rendered feasible and physiologically relevant a study of the effect of the daily intravenous administration of a mixture of these materials in quantities of these orders of magnitude. The neutral environment of the alimentary tract normally required for the potentiation of beef muscle by gastric juice upon oral administration[12] is presumably compatible with an effective interaction between intrinsic factor and vitamin B_{12} upon parenteral administration under the conditions employed.

Observations designed to determine whether vitamin B_{12} is rendered more potent upon intravenous administration by contact with intrinsic factor were satisfactorily completed upon six patients according to the following plan. For use during the first periods of observation a mixture of inactivated gastric juice and vitamin B_{12} was prepared each day as follows: ten milliliters of sterile gastric juice from the pool were boiled for five minutes on a water bath in a vial, through the rubber top of which liquids could be inserted aseptically by use of a syringe and hypodermic needle. Immediately thereafter the vial was cooled and 1 ml. of a sterile aqueous solution containing 0.33 μg of vitamin B_{12} was introduced. The resulting mixture was then incubated for one hour at 37.5° C. Thereupon it was quantitatively added to 150 ml. of sterile normal saline solution and given to the patient by intravenous injection during a period of from fifteen to twenty minutes. During the second period the daily procedure was identical except that the sterile gastric juice derived from the same pool was *not* inactivated by boiling prior to the addition of the vitamin B_{12} solution. The effects of these procedures upon erythropoiesis are shown in Table I. During the first periods of observation each of five patients, Cases 130, 131, 132, 133, and 134, given 0.33 μg of vitamin B_{12} and 10 ml. of boiled gastric juice exhibited slight or moderate reticulocyte responses. Four of these patients, Cases 130, 131, 132, and 133, showed no convincing second reticulocyte peaks when unboiled gastric juice was substituted during the second periods of observation. The slight numerical increase of the reticulocytes during the second periods of Cases 130 and 132 cannot be distinguished with certainty from the prolonged moderate elevation of reticulocytes that sometimes accompanies the uniform daily administration of a weakly hematopoietic substance. Certainly the responses in Cases 130 and 132, if such they are, are not comparable in magnitude to those demonstrating the enhancement of the effect of 1 μg of vitamin B_{12} by 10 ml. of gastric juice when both are given daily by mouth (see Table II, Case 136, first period). The fifth patient, Case 134, showed a definite, indeed an even larger, reticulocyte peak during the second period. A sixth patient, Case 135, who received only 0.25 μg of vitamin B_{12} daily, failed to exhibit a reticulocyte response in either period. Cases 131 and 132 showed definite second reticulocyte peaks when in the third periods of observation the daily dose of

TABLE I. FAILURE OF GASTRIC (INTRINSIC) FACTOR DEMONSTRABLY TO INCREASE THE HEMATOPOIETIC ACTIVITY OF VITAMIN B_{12} UPON SIMULTANEOUS INTRAVENOUS ADMINISTRATION

DAYS OF TREATMENT	CASE 130		CASE 131		CASE 132		CASE 133		CASE 134		CASE 135*	
	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)
First periods: Daily intravenous administration of 0.33 μg vitamin B_{12} and 10 ml. sterile boiled gastric juice												
0	1.40	0.5	2.30	0.2	1.68	1.7	1.07	1.6	1.95	0.4	1.50	0.8
2		1.3	2.05	0.2	1.68	1.4		1.0	1.65	0.1	1.25	0.5
4	1.35	1.3		0.9		1.4	1.15	2.3		0.3		0.7
6		5.7	1.64	0.3	1.48	5.1	1.21	5.0	1.67	0.9	1.53	0.7
8	15.8	**11.3**	1.52	1.2	1.64	8.4		7.1	1.48	6.6		**1.9**
10	2.01	10.9		2.9		**15.6**	1.49	4.3		4.6	1.41	1.1
12		5.7	1.62	**3.3**	1.73	9.4	1.83	9.0	1.84	**6.3**		
14	1.76	4.9			1.93	**6.7**		**11.9**	1.48	2.7		
16						**5.1**	1.89	10.1	1.60	2.6		
18					**1.85**	**5.2**	2.20	6.7				
Second periods: Daily intravenous administration of 0.33 μg vitamin B_{12} and 10 ml. sterile unboiled gastric juice												
2	1.87	5.2	1.63	0.5	**1.89**	**3.8**	2.31	7.4		1.6		1.5
4		7.8		1.3		**3.5**		2.5	1.75	**4.1**	1.26	1.0
6	2.00	8.3	1.57	0.7	2.28	4.8	2.23	2.0		**7.1**	1.17	1.1
8		8.6	1.40	1.1	2.47	5.1		0.8	1.70	**9.2**		1.2
10	2.20	5.6	1.34	0.5	2.43	1.4	2.12	1.2		5.6	2.13	1.1
12	2.41	4.3							2.12	2.5		
Third periods: Daily intramuscular administration of vitamin B_{12} in amounts indicated												
			1.0 μg		1.0 μg		0.66 μg				> 5.0 μg	
2			1.38	0.9		1.1		2.0				1.1
4				1.4	2.42	1.4	2.30	1.7			1.64	0.9
6			1.52	4.3		9.8	1.64	2.8			1.49	1.0
8			1.53	7.7	2.52	**10.7**		3.0				**2.5**
10				**9.1**	**2.78**	7.7	2.61	1.5			1.81	10.9
12			1.95	8.1							2.01	**28.1**
14			1.98	11.1								15.8

Rules at bottom of individual columns of figures indicate when one period of therapy ended and a subsequent period began.

*Case 135 received 0.25 μg instead of 0.33 μg of vitamin B_{12} in the first two periods.

vitamin B_{12} was increased to 1 μg intramuscularly. However, Case 133 did not so respond when the daily dose of vitamin B_{12} was increased to only 0.66 μg. Case 135 showed a striking first reticulocyte response in the third period only when receiving an average of more than 5 μg of vitamin B_{12} intramuscularly daily. Thus, with the exception of Case 134, these data lend no convincing support to the idea that contact with intrinsic factor enhances the potency of vitamin B_{12} upon parenteral administration.

Does a stoichiometric relation exist between vitamin B_{12} and intrinsic factor with respect to the potentiation of vitamin B_{12} by intrinsic factor upon simultaneous oral administration?—The ability of gastric juice to render vitamin B_{12} unavailable for the support of microbial growth has been stated to be directly related to the quantity of a given sample of gastric juice employed[8]; and it has been suggested that this binding capacity is in some way closely related to the essential physiologic action of intrinsic factor.[13] Although sufficient evidence[14-16] has now appeared to render this assumption doubtful, it nevertheless seemed

TABLE II. RECIPROCAL RATHER THAN STOICHIOMETRIC RELATION BETWEEN AMOUNTS OF VITAMIN B_{12} AND INTRINSIC FACTOR REQUIRED FOR A GIVEN ERYTHROPOIETIC EFFECT UPON SIMULTANEOUS ORAL ADMINISTRATION

DAYS OF TREATMENT	CASE 136*		CASE 137		CASE 138		CASE 139	
	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)
First periods: Daily oral administration of substances indicated 1 μg vitamin B_{12} and 10 ml. gastric juice								
0	1.70	1.0	1.39	4.8	1.52	3.8	1.58	0.8
2	1.71	0.8		1.8	1.49	3.0	1.73	1.0
4		0.3	1.48	1.8		0.6		1.1
6		1.0		2.2	1.55	0.6	1.57	2.1
8	1.49	6.9	1.35	2.7		0.2	1.70	6.0
10	1.64	**10.0**	1.69	5.7	1.46	1.4	1.74	8.2
12		6.4		4.6				**13.0**
14	1.97	2.6	1.56	**6.1**			2.12	3.7
Second periods: Daily oral administration of substances indicated								
5 μg vitamin B_{12} and 10 ml. gastric juice					1 μg vitamin B_{12} and 25 ml. gastric juice		50 ml. gastric juice	
2	1.65	1.6	1.58	3.4	1.30	0.9		3.5
4		1.1	1.79	3.0	1.18	2.4	2.15	4.3
6	1.65	1.0		5.7		2.4		4.7
8	1.75	**4.7**	2.10	14.2	1.28	4.0	2.33	9.2
10		**6.1**	2.30	**18.0**	1.36	4.7	2.18	**10.0**
12	2.01	**7.7**		4.2				4.4
14		6.1	2.40	3.3				
Third periods: Daily intramuscular administration of substance indicated 1 μg vitamin B_{12}								
2	1.98	3.8				2.7	2.25	2.7
4	2.47	3.8			1.40	1.9	2.40	2.5
6		2.7			1.39	3.7	2.37	2.7
8	2.61	**10.0**			1.56	8.0		**5.3**
10		6.6				11.9	3.21	4.4
12	2.96	2.9			1.92	**13.3**		1.9
14						9.1		
16					2.09	9.3		

Rules at bottom of individual columns of figures indicate when one period of therapy ended and a subsequent period began.

*Case 136 received 1 μg of vitamin B_{12} orally daily without detectable erythropoietic effect during the ten days immediately preceding the first period shown above.

of interest to determine whether or not the clinical response, like the in vitro binding effect of gastric juice upon vitamin B_{12}, involved a direct quantitative relation between these substances.

Observations designed to answer this question were satisfactorily completed upon four patients. The effects of the procedures upon erythropoiesis are shown in Table II. During the first periods of observation on two patients, Cases 136 and 137, 1 ml. of a solution containing 1 μg of vitamin B_{12} and 10 ml. of normal human gastric juice were mixed and at once given daily by mouth. The small flask which had contained the mixture was carefully rinsed with water, which the patient then drank. Moderate reticulocyte responses appeared in both instances. During the second periods the amount of vitamin B_{12} was increased to 5 μg a day without other change in the procedure. In both patients distinct second reticulocyte responses developed. Two other patients, Cases 138 and 139, also received during the first periods of observation 1 μg of vitamin B_{12} and 10 ml. of gastric juice. In Case 138 there was no

reticulocyte response, but in Case 139 a definite reticulocyte peak developed. During the second periods the same procedure was followed except that the daily amount of gastric juice was increased, in Case 138 to 25 ml., and in Case 139 to 50 ml. In both patients definite reticulocyte responses then appeared. During the third periods of observation on Cases 136, 138, and 139 reticulocyte responses again appeared when 1 μg of vitamin B_{12} was given daily by intramuscular injection. Thus, apparently at least with the quantities employed here, upon simultaneous daily oral administration an increase in either the amount of vitamin B_{12} or of gastric juice produces a greater hematopoietic effect. However, this response is still not so great as that observed from the subsequent injection of the vitamin B_{12} alone.

Does the order of the serial oral administration of vitamin B_{12} and of intrinsic factor influence the hematopoietic effect of the vitamin B_{12}?—The results presented in the first section of this article give no consistent indication that the hematopoietic activity of vitamin B_{12} is enhanced upon simultaneous intravenous administration with intrinsic factor. This and other clinical observations[14-16] yield no evidence that this involves a reaction between vitamin B_{12} and intrinsic factor. Instead, the enhanced activity of orally administered vitamin B_{12} when given together with gastric juice is presumably merely the result of its increased absorption, a process which, as shown in the observations on Cases 136, 138, and 139, does not approach the efficiency of parenteral injection of vitamin B_{12} alone. It is therefore possible that intrinsic factor has a specific effect upon the permeability of the intestinal wall to vitamin B_{12}, in which a relation with vitamin B_{12} may be only secondarily involved. Consistent with such a hypothesis would be evidence indicating that prior contact of intrinsic factor with the wall of the alimentary tract rendered vitamin B_{12} when subsequently administered more active than when, *with equal opportunity for contact between the two substances,* administration of vitamin B_{12} preceded that of intrinsic factor. A mutual influence between these substances is not observed when an interval of as long as twelve hours intervenes between their separate oral administration.[1] However, an effect may be detected, at least with beef muscle and gastric juice, when the interval between their separate administration is as short as six hours.[17] Consequently, a plan of procedure was established in which, during a first period of observation, 5 μg of vitamin B_{12} was given daily in 50 or 75 ml. of water by mouth at 8 P.M. and was followed by an equal amount of neutralized gastric juice at 11 P.M. or at midnight. During a second period of observation the same amounts of these substances and the same time interval were used, but the order of administration was reversed. During a third period the gastric juice and vitamin B_{12} were given simultaneously.

In Table III are shown the results obtained in five patients. In four of these patients, Cases 140, 141, 143, and 144, reticulocyte responses appeared during the first periods of observation. In two, Cases 140 and 141, responses again appeared in the second periods, indicating the greater hematopoietic activity of the vitamin B_{12} when it was preceded by gastric juice. A similar conclusion derives from the observations on Case 142, in which a reticulocyte response appeared for the first time during the second period. In Cases 143 and

144 in which large, perhaps maximal, initial reticulocyte responses relative to initial red cell levels developed during the first periods, no second responses appeared either in the second periods or in the third periods of observation when the gastric juice and vitamin B_{12} were given together. From these two cases no conclusions can be drawn. Under similar therapy, however, additional reticulocyte responses developed in the third periods of observation of Cases 140, 141, and 142. Thus, it appears that when vitamin B_{12} and intrinsic factor are given a similar but limited opportunity for mutual contact upon oral administration, the potency of vitamin B_{12} may be detectably more enhanced when, as in Cases 140, 141, and 142, the administration of intrinsic factor precedes by a few hours that of vitamin B_{12} than when this order is reversed.

DISCUSSION

From the results reported in the second section of this article, in observations on Case 136 (including a preliminary period not shown in Table II), it would appear that the hematopoietic effect of 1 μg of vitamin B_{12} is distinctly increased by the simultaneous daily oral administration of 10 ml. of gastric juice. However, even the effect of 5 μg of vitamin B_{12} and 10 ml. of gastric juice (Case 136) or of 1 μg of vitamin B_{12} and 25 or 50 ml. of gastric juice (Cases 138 and 139) does not equal that of the daily injection of 1 μg of vitamin B_{12} alone. Horrigan and his associates[5] have now shown that vitamin B_{12} is itself directly effective in causing *local* maturation of megaloblasts upon injection into the bone marrow cavity in pernicious anemia; and we have confirmed this in one patient. Thus, it is probable that the physiologic action of intrinsic factor is merely to promote the absorption of vitamin B_{12}.[1, 2] Directly confirmatory of this hypothesis is the recent demonstration by Welch and his associates[6] that intrinsic factor reduces significantly the fecal excretion of radioactive material when given by mouth simultaneously with radioactive cobalt-containing vitamin B_{12}.

The unlikely alternative or, conceivably, additional action of intrinsic factor upon vitamin B_{12}, namely, the formation of a hematopoietically more active complex, at least receives no consistent support from the observations reported in the first section of this article in which incubated mixtures of vitamin B_{12} and sterile gastric juice were given intravenously. It should be pointed out, however, that the so-called method of serial reticulocyte responses[10] employed in making these observations provides proof of the greater hematopoietic activity of a given substance *only when positive*, that is, only when the substance in question causes a reticulocyte response. Thus, the absence of a second reticulocyte response in five of six observations,* although consistent with a lack of increase in activity of the vitamin B_{12} during the second period when it was given with intrinsic factor, obviously does not provide positive proof that such is the case (Table I). However, if the formation of a more active combination was responsible to any significant degree for the potentiating effect of intrinsic factor on vitamin B_{12}

*A different batch of gastric juice was employed during the second period of Case 134 during which a second reticulocyte response appeared. However, because of the virtual lack of vitamin B_{12} in Berkefeld-filtered gastric juice,[18] it seems unlikely that this defect of the experimental procedure resulted in the administration of a significantly larger amount of vitamin B_{12} during the second period.

TABLE III. GREATER HEMATOPOIETIC EFFECT OF ORALLY ADMINISTERED VITAMIN B_{12} WHEN PRECEDED RATHER THAN FOLLOWED BY ORALLY ADMINISTERED INTRINSIC FACTOR

DAYS OF TREATMENT	CASE 140		CASE 141		CASE 142*		CASE 143		CASE 144	
	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)	R.B.C. ($\times 10^6$)	RETICS. (%)
	First periods: Daily oral administration of substances as indicated									
	8 P.M.: 5 μg vitamin B_{12} 12 M. : 75 ml. gastric juice		8 P.M.: 5 μg vitamin B_{12} 11 P.M.: 75 ml. gastric juice				8 P.M.: 5 μg vitamin B_{12} 11 P.M.: 50 ml. gastric juice			
0	1.53	1.1	1.53	2.0	2.09	0.4	1.15	1.5	2.21	0.1
2	1.39	0.5		0.3	2.04	0.5		2.7	2.14	0.1
4	1.52	0.4	1.60	1.1		0.2	1.37	3.5		0.2
6		1.1	1.51	3.7	2.14	0.2	1.30	4.6	2.23	0.6
8		3.6		**16.8**		0.6		12.7	2.06	3.7
10	1.35	3.2	1.83	10.8	2.00	0.1	1.50	**20.0**		4.1
12	1.42	5.5	1.81	2.6			1.72	4.3	2.37	**6.4**
14	1.57	**6.7**						3.8		4.2
16	1.69	3.1								
	Second periods: Daily oral administration of substances as indicated									
	8 P.M.: 75 ml. gastric juice 12 M. : 5 μg vitamin B_{12}		8 P.M.: 75 ml. gastric juice 11 P.M.: 5 μg vitamin B_{12}				8 P.M.: 50 ml. gastric juice 11 P.M.: 5 μg vitamin B_{12}			
2		1.9		3.5		0.4	1.86	2.7	2.39	1.9
4	1.80	1.8	1.68	3.2	2.11	2.6	1.90	3.2		2.7
6	1.79	1.2		2.6		4.6		1.5	2.62	1.7
8		2.1	1.84	7.4	2.37	**7.6**	1.87	1.1		0.5
10	1.76	2.4	2.09	**8.6**	2.29	3.2	2.11	1.2	2.71	1.9
12		3.3	2.14	5.6					3.19	1.0
14	1.87	5.2								
16	2.26	**6.4**								
18		4.7								
20	2.29	1.1								

	Third periods: Daily oral administration of substances as indicated									
	12 M. : 25 ml. gastric juice and 5 μg vitamin B_{12} together		8 P.M.: 75 ml. gastric juice and 5 μg vitamin B_{12} together				8 P.M.: 50 ml. gastric juice and 5 μg vitamin B_{12} together			
2	2.62	1.5	2.29	3.9	2.30	1.9		1.8	3.08	1.0
4		1.0		3.5		2.0	2.22	1.5	3.01	0.9
6	2.50	1.0	2.47	3.0	2.32	1.2		3.3		2.0
8		2.0		3.8	2.41	5.0	2.32	1.2	3.35	1.6
10	2.90	3.4	2.64	**6.8**		**12.9**		2.4		1.0
12	3.01	**4.2**	2.73	5.7	2.83	7.9			3.46	1.4
14		3.4								
16	3.15	2.3								

Rules at bottom of individual columns of figures indicate when one period of therapy ended and a subsequent period began.
*Case 142 had total gastrectomy four and one-half years previously.

when given by mouth, such an increase in hematopoietic activity should be even more apparent upon parenteral administration of the combination. Clearly, as is seen from the reticulocyte responses in the third periods of Cases 131 and 132, the hematopoietic activity of 0.33 μg of vitamin B_{12} and 10 ml. of sterile gastric juice given parenterally is not so great as that of 1 μg of vitamin B_{12} so given alone.

Nevertheless, a possible objection to this interpretation can be based on the work of Callender and Lajtha.[9] It may be argued that if their so-called "extra-gastric intrinsic factor" exists in the serum of the patient, the vitamin B_{12} when injected alone would combine with it and so be as active as when given in equal quantity with the "gastric intrinsic factor" as here. However, the existence of an effective reaction between vitamin B_{12} and the "extra-gastric intrinsic factor" of the patient's serum requires postulation of yet another metabolic disturbance because according to Callender and Lajtha[9] "B_{12} added to pernicious anemia serum has no megaloblast ripening effects in vitro" as does normal serum. For the present, therefore, we prefer the simpler working hypothesis based on the established clinical facts[1, 2, 5, 6] referred to in the first paragraph of this discussion. This hypothesis represents the problem as one of transporting vitamin B_{12} in fundamentally unaltered form across the intestinal barrier. Thereafter the blood stream carries it to the bone marrow.

The observations described in the second section of this article, indicating that an increase in the amount either of vitamin B_{12} or of intrinsic factor enhances the hematopoietic effect of their simultaneous oral administration, confirm the experience of Glass and his associates[19] on this point. The seemingly reciprocal character of the quantitative relation between these substances in this essential physiologic action of intrinsic factor (Table II) throws further doubt on the relevance to this process of the stoichiometric relation between gastric juice and vitamin B_{12}[8] described with respect to inhibition of the growth of vitamin B_{12} requiring bacteria.[13] Actually, such binding capacity exhibits but poor correlation with the intrinsic factor activity of fractions of gastric mucosa.[14, 15] Moreover, a certain amount of binding power for vitamin B_{12} is the property of various proteins including lysozyme;[20] and finally, such binding power is stated to be present in normal saliva,[13] a secretion which is known *not* to be a source of intrinsic factor.[21]

These and other observations[16, 22] have thus far failed to demonstrate that any reaction between intrinsic factor and vitamin B_{12} provides the basis of the manifest effect of intrinsic factor in increasing the hematopoietic activity of vitamin B_{12} upon oral administration in pernicious anemia. If, however, the function of intrinsic factor is merely to promote the absorption of vitamin B_{12} by the alimentary tract, its measurable influence should perhaps be sought in an effect of intrinsic factor, not upon vitamin B_{12}, but upon a specific absorbing mechanism of the bowel. Consequently, an attempt was made to discover whether the order of serial administration of vitamin B_{12} and of intrinsic factor made a difference in the apparent hematopoietic effectiveness of the vitamin B_{12}. It was reasoned that if the essential function of the intrinsic factor is, so to speak, to prepare the small intestine for the reception of the vitamin B_{12}, the adminis-

tration of intrinsic factor a short time before the administration of vitamin B_{12} might produce more active hematopoiesis than if these substances were given in the reverse order when separated by a similar time interval, which presumably would allow a similar degree of contact between them.

As described in the third section of this article, in three of the five patients studied, Cases 140, 141, and 142 (Table III), the prior administration of intrinsic factor appeared to be more effective hematopoietically than the prior administration of vitamin B_{12} when these substances were given separately with an interval of either three or four hours. No conclusion can be drawn as to the relative effectiveness of the order of serial administration of these substances in the other two patients, Cases 143 and 144. In them even the subsequent simultaneous administration of vitamin B_{12} and intrinsic factor during the third periods did not produce second reticulocyte responses following those seen in the first periods. Earlier clinical observations[12] indicate that acidified, buffered mixtures of beef muscle and gastric juice are hematopoietically inert until made more nearly neutral. This we interpret today to mean that vitamin B_{12} is absorbed from the upper reaches of the gut, where the reaction of the intestinal milieu would be most effectively altered. A similar conclusion with regard to the locus of absorption of vitamin B_{12} is suggested by the fact that in the pernicious tapeworm anemia of Finland, displacement of the parasite from the upper to the lower ileum sometimes permits a remission to occur.[23] It is of interest with respect to Case 142 that the patient's entire stomach had been surgically removed four and one-half years previously. Hence if no significant interaction between vitamin B_{12} and intrinsic factor is assumed to occur, we may possibly infer that the fundamental action of intrinsic factor is on a superficially located hypothetical structure or chemical process in the wall of the upper small intestine. Other interpretations are, of course, possible. The effect observed here may mean nothing more than that the natural rate of passage of intrinsic factor through the alimentary tract is slower than that of vitamin B_{12}. If so, a greater degree of contact between these agents would occur when, with the gastric juice administered first, it was "overtaken" by the faster moving vitamin B_{12}. However, slow progress of intrinsic factor might in fact imply a delay in passage due to its avidity for contact with the intestinal wall, there resulting in an effect which in turn might temporarily facilitate the absorption of vitamin B_{12}.

SUMMARY AND CONCLUSIONS

Observations were made upon fifteen patients with pernicious anemia under controlled dietary conditions. No consistent evidence was obtained for potentiation of the hematopoietic effect of vitamin B_{12} by simultaneous daily intravenous injection with intrinsic factor. A reciprocal rather than a stoichiometric relation exists between vitamin B_{12} and intrinsic factor with respect to the potentiation of vitamin B_{12} by intrinsic factor upon simultaneous daily oral administration.

The hematopoietic effect of the daily oral administration of 1 μg of vitamin B_{12} may be detectably increased by as little as 10 ml. of normal human gastric juice. However, the hematopoietic effect of 1μg of vitamin B_{12} and 50 ml. of gas-

tric juice does not equal that of the daily injection of 1 μg of vitamin B_{12} alone. When separated by an interval of three or four hours the hematopoietic effect of the *serial* administration of vitamin B_{12} and of intrinsic factor is greater when the intrinsic factor precedes the vitamin B_{12}. These and other recent clinical observations suggest that the essential physiologic function of intrinsic factor is merely to increase somewhat the assimilation of vitamin B_{12}. They indicate the possibility that the primary effect of intrinsic factor is upon the intestinal wall rather than upon the vitamin B_{12}.

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FURTHER STUDIES ON THE METABOLISM OF L-TYROSINE IN SCORBUTIC MONKEYS

The Effect of Folinic Acid, Cortisone, Adrenalectomy, and Sulfhydryl Groups on Urinary Excretion of p-hydroxy-phenyl Compounds

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THE excretion in the urine of p-hydroxy-phenyl compounds (tyrosyluria) in relation to the metabolism of tyrosine has been the subject of many investigations. Studies in this laboratory have been directed toward the tyrosyluria produced in scorbutic monkeys by the administration of a dose of L-tyrosine. The object of this report is to present additional data extending observations previously reported.[1, 2]

The phenomenon of tyrosyluria may appear under natural circumstances in premature infants[3-8] and in pernicious anemia in relapse.[9, 10] On the other hand it may be induced in relatively unnatural circumstances by the administration of large doses of L-tyrosine to scorbutic guinea pigs,[11-19] scorbutic infants,[20] and to human adults with scurvy.[21] The discovery of the restorative effect of pteroylglutamic acid (PGA) on the oxidation of tyrosine by liver slices from PGA deficient rats[22, 23] and the demonstration[18, 19] that PGA was effective in abolishing the induced tyrosyluria in scorbutic guinea pigs suggested that PGA was also involved. But PGA was not effective in the scorbutic monkey.[1] When it was shown[24] that the folinic acid demonstrable in liver slices of PGA deficient rats after incubation with PGA was greatly increased by the presence of ascorbic acid in the medium, it was thought possible that the failure of PGA in the scorbutic monkey to abolish the tyrosyluria might be due to lack of folinic acid formation. However, folinic acid was shown to be without effect on the induced tyrosyluria in the scorbutic monkey.[2]

This paper reports the similar failure of more persistent efforts to prevent tyrosyluria in the monkey with folinic acid. It was also reported[2] that cortisone given before and during tyrosine loading prevented tyrosyluria in scorbutic monkeys. The present paper reports two further tests with cortisone, in one of which cortisone failed to reduce the tyrosyluria and in one it was partially successful in reducing but not abolishing it. This agrees with the variable results obtained with cortisone by others.[25] The final test as to whether failure of the adrenal cortex might be the cause of the tyrosyluria, as has been suggested,[26] would be to adrenalectomize an animal and then test for tyrosyluria. This has been done and no spontaneous tyrosyluria resulted,

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TABLE I. EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS IN THE URINE OF MONKEYS LOADED WITH L-TYROSINE (2 GM. PER KILOGRAM) AND TREATED WITH VARIOUS DRUGS

MONKEY NUMBER	WT. KG.	THERAPY	AVERAGE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS GM./24 HR.	AVERAGE PER CENT OF THE DAILY LOAD OF TYROSINE
10 controls with adequate ascorbic acid				(Av.) 12.8
16 scorbutic monkeys				(Av.) 55.2
113	1.68	Folinic acid,* 2 mg. for 11 days	2.05	57.8
108	2.4	Folinic acid, 1 mg. b.i.d. for 4 days	1.96	39.2
108	2.4	Cortisone acetate, 50 mg. for 5 days,† 75 mg. for 3 days	2.98	59.5
86	1.45	Cortisone acetate, 25 mg. b.i.d. for 3 days	.71	24.6
113	1.68	Cortisone acetate, 10 mg. once a day for 2 days	1.70	49.9
69	1.3	ACTH,‡ 10 mg. b.i.d. for 2 days	1.75	67.5
78	2.33	ACTH, 10 mg. t.i.d. for 4 days	2.59	54.0
198	2.4	ACTH, 10 mg. b.i.d. for 3 days, 20 mg. b.i.d. 2 days	2.84	56.8
79	2.4	Cysteine, 200 mg. b.i.d. 1 day —q.i.d. 1 day, 400 mg. t.i.d. 1 day	1.62	33.7
116	2.45	Glutathione, 500 mg. b.i.d. 5 days	.92	18.7
78	2.33	Glutathione, 100 mg. t.i.d. 4 days	2.28	47.6
117	2.19	BAL, 20 mg. b.i.d. 3 days, 20 mg. q.i.d. 1 day, 20 mg. t.i.d. 3 days	1.00	22.6
164	1.54	D-ascorbic acid,§ 50 mg./day 12 days, 50 mg./day 19 days	1.79 1.21	57.6 35.6

*Generously provided by Dr. Edward Campbell, Director of Division of Biochemical Research, Eli Lilly Research Laboratories. Indianapolis, Ind., where it was synthesized and assayed.

†Cortone-Merck and Company, Inc., Rahway, N. J.

‡Generously supplied through the courtesy of Dr. E. E. Hays, Director of the Medical Research Laboratories, Armour and Company, Chicago, Ill.

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nor could it be induced by giving a load of tyrosine. The animal was supplied with adequate ascorbic acid.

There is evidence in the literature that ascorbic acid has an effect on the amount of free sulfhydryl groups present in tissues[27-29] and that these compounds play a role in many enzyme systems of the body.[30] Whether ascorbic

acid functions in preventing tyrosyluria by stabilizing some enzyme whose activity depends on its containing free sulfhydryl group might be tested in two ways. Compounds containing free sulfhydryl groups might be administered to scorbutic animals with induced tyrosyluria in an attempt to abolish it, or a sulfhydryl antagonist might be given to a nonscorbutic animal in an attempt to produce tyrosyluria when a tyrosine load was given.

Both these approaches have been tried and have failed to demonstrate that a sulfhydryl enzyme is involved.

EXPERIMENTAL

Rhesus monkeys were fed on various milk diets as described in previous papers which may be consulted for detailed information concerning the diets and manifestations of the animals.[31, 32] The protein intake was at least 3 Gm. per kilogram of body weight. Fifty milligrams of ascorbic acid was given daily to the controls. In most cases when used for the loading tests the monkeys were very scorbutic, but in one or two cases, though definitely ascorbic acid deficient, signs of scurvy had not yet developed. In all cases the method of applying the loading test was to mix L-tyrosine (2 Gm. per kilogram of body weight) with 50 to 100 ml. of the milk formula and administer it by gavage.

The urine was collected in bottles containing 14N. sulfuric acid (2 ml. for each 100 ml. of urine expected) the twenty-four hour period being reckoned from the time of the administration of the load until just before giving the load the next day. The twenty-four hour specimens were measured and analyzed for p-hydroxy-phenyl derivatives by Millon's test as modified by Folin and Ciocalteu and adapted by Medes.[33]

TABLE II. EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS ON LOADING WITH L-TYROSINE BEFORE AND AFTER TOTAL ADRENALECTOMY

ANIMAL 52 (WT.)	DAY	LOAD OF TYROSINE (GM.)	TOTAL P-HYDROXY-PHENYL COMPOUNDS (GM./24 HR.) EXCRETED	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
3.25 kg.	0	0	0.035	- -
	1	6.7	0.337	5.0
	2	6.7	0.593	6.9
	3	6.7	0.455	6.9
	4	0	0.517	9.5
	5	0	0.055	- -
	17	Bilateral adrenalectomy		
3.4	27	0	0.045	- -
	28	6.8	0.161	2.4
	29	6.8	0.31	4.6
	30	6.8	0.27	4.0
2.8	57	0	0.064	- -
	58	5.6	0.377	6.7
	59	5.6	0.744	13.3
	60	5.6	1.25	22.2
3.0	182	0	0.087	- -
	183	6.0	0.737	12.3
	184	6.0	specimen lost	- -
	185	6.0	0.481	8.0
	186	6.0	0.449	7.5
	187	6.0	0.558	9.3
	188	6.0	0.415	9.3
	189	6.0	0.233	3.9
	190	0	0.095	- -
	191	0	0.049	- -

RESULTS

Tables I to III in the text give summaries of the results obtained in the various groups of animals. The detailed data concerning treatment and results in the individual animals are given consecutively in Tables IV to XV as an appendix.

The excretion of p-hydroxy-phenyl compounds is given in grams per twenty-four hours, calculated as though they were tyrosine, which was used in setting up the standard curves for spectrophotometric measurements.

Effect of Folinic Acid.—In monkey 113, which had received 2 mg. of folinic acid for seven days before loading with tyrosine was commenced, the tyrosyluria was as marked as in an untreated scorbutic animal. In monkey 108, which had received ACTH and cortisone for sixteen days without effect on the tyrosyluria before folinic acid was started, there was ultimately a decrease in the tyrosyluria after 7 mg. of folinic acid had been administered. It is impossible to say whether this was due to the folinic acid or to the combined treatment with cortisone and folinic acid.

Effect of Cortisone and ACTH.—In monkey 108 which had been given ACTH without abolition of the tyrosyluria for four days prior to the use of cortisone there was actually an increase in the tyrosyluria when cortisone was given. In monkey 113 in which tyrosyluria had persisted for four days in spite of intensive folinic acid therapy there was a fall in the excretion of p-hydroxy-phenyl compounds the second day of cortisone therapy. In monkey 86 four days of intensive ACTH therapy had failed to produce any drop in the tyrosyluria. On the day of the change to cortisone therapy there was a drop in the urine volume and total excretion of p-hydroxy-phenyl compounds actually decreased before the cortisone was given. This was maintained during the cortisone therapy. Whether this was due to exhaustion of the animal by its long ordeal of treatment it is impossible to say. It would seem that ACTH is not effective in reducing the tyrosyluria of scorbutic monkeys loaded with L-tyrosine but that cortisone may be.

TABLE III. EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS IN THE URINE OF NONSCORBUTIC MONKEYS WHEN LOADED WITH L-TYROSINE (2 GM. PER KILOGRAM) AND GIVEN INJECTIONS OF SULFHYDRYL INHIBITORS

MONKEY NO.	WT. (KG.)	THERAPY	AVERAGE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS GM./24 HR.	PER CENT OF THE DAILY LOAD OF TYROSINE
10 controls with adequate ascorbic acid				12.8 (Av.)
16 scorbutic				55.2 (Av.)
115	2.0	Iodoacetate 40-120 mg./day	0.41	10.3
33	3.9	Iodoacetate 80 mg. 4 day 160 mg. 3 day	0.55	7.0
151	3.2	P-chloromercuribenzoate 4-40 mg. i.m. and 100 mg. orally	0.47	10.3

TABLE IV. MONKEY 33, WEIGHT 3.9 KG. RECEIVING ASCORBIC ACID AND LOADED WITH L-TYROSINE (2 GM./KG.) EFFECT OF IODOACETATE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0		--	0.04	--
1	0900	Tyrosine 7.8 Gm.	0.31	4.0
2	0930	Tyrosine 7.8 Gm.		
3	0930	Tyrosine 7.8 Gm.	0.56	7.2
	1600	IAA 80 mg.		
4	0900	Tyrosine 7.8 Gm.	0.56	7.2
	1600	IAA 80 mg.		
5	0900	Tyrosine 7.8 Gm.	0.45	5.7
	1600	IAA 80 mg.		
6	0800	Tyrosine 7.8 Gm.	0.49	6.2
	1450	IAA 80 mg.		
7	0800	IAA 80 mg.	0.64	8.2
	0900	Tyrosine 7.8 Gm.		
	1600	IAA 80 mg.		
8	0800	IAA 80 mg.	0.65	8.3
	0900	Tyrosine 7.8 Gm.		
	1600	IAA 80 mg.		
9	0800	IAA 80 mg.	0.50	6.4
	0900	Tyrosine		
	1600	IAA 80 mg.		
10	0800	IAA 80 mg.		
	0900	Tyrosine 7.8 Gm.		
11		Died		

Effect of Sulfhydryl Compounds.—Monkey 79 was scorbutic and showed pronounced tyrosyluria on loading with L-tyrosine. It was then given intramuscular injections of a 10 per cent aqueous solution of cysteine in increasing doses according to the schedule shown in Table VII. There was a reduction of the tyrosyluria on the second day of administration of the cysteine, but the injections proved too toxic and the animal died on the fourth day.

Next an aqueous solution of glutathione was tried. Monkey 78 had been loaded with L-tyrosine for eight consecutive days and had a well-established

TABLE V. MONKEY 19. WEIGHT 1.3 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KG.) EFFECT OF CORTICOTROPIN ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
1	0900	ACTH 10 mg.	1.86	71.5
	1000	Tyrosine 2.6 Gm.		
	1700	ACTH 10 mg.		
2	0900	ACTH 10 mg.	1.65	63.5
	1000	Tyrosine 2.6 Gm.		
	1700	ACTH 10 mg.		

TABLE VI. MONKEY 78. WEIGHT 2.33 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KILO.) EFFECT OF CORTICOTROPIN AND GLUTATHIONE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	----	----	0.02	----
1	1700	Tyrosine 4.8 Gm.	0.81	16.9
2	1700	Tyrosine 4.8 Gm.	2.56	53.4
3	1600	ACTH 10 mg.	3.13	65.2
	1700	Tyrosine 4.8 Gm.		
	2200	ACTH 10 mg.		
4	0600	ACTH 10 mg.	3.19	66.4
	1200	ACTH 10 mg.		
	1600	Tyrosine 4.8 Gm.		
	1800	ACTH 10 mg.		
	2300	ACTH 10 mg.		
5	0700	ACTH 1 mg.	2.09	43.5
	1100	ACTH 8 mg.		
	1600	ACTH 10 mg.		
	1700	Tyrosine 4.8 Gm.		
	2300	ACTH 10 mg.		
6	0700	ACTH 10 mg.	2.27	47.3
	1600	ACTH 24 mg.		
	1700	Tyrosine 4.8 Gm.		
7	1700	Tyrosine 4.8 Gm.	2.29	47.7
8	1700	Tyrosine 4.8 Gm.	2.97	61.8
9	1500	Glutathione 500 mg. (GSH) i.m.	2.70	51.0
	1600	Tyrosine 4.8 Gm.		
	1800	GSH 500 mg.		
10	0700	GSH 500 mg.	2.45	51.0
	1500	GSH 500 mg.		
	1700	Tyrosine 4.8 Gm.		
	2300	GSH 500 mg.		
11	0700	GSH 500 mg.	1.00	21.0
	1500	GSH 500 mg.		
	1700	Tyrosine 4.8 Gm.		
	1900	GSH 500 mg.		
12	0700	GSH 500 mg.	1.30	27.0
	1700	Tyrosine 4.8 Gm.		
	1800	GSH 500 mg.		
13	1900	Died		

tyrosyluria which had not been abolished by injections of ACTH totaling 114 mg. over a period of four days. On the ninth day of loading, injections of an aqueous solution of glutathione were commenced and continued for four days (Table VI) (3.5 Gm. of glutathione were suspended in 25 ml. of distilled water and 4 ml. of 0.1N HCl added and stirred until dissolved. The final volume was made up to 35 ml.). Again toxic reactions occurred and the animal died on the thirteenth day. There was a reduction in the tyrosyluria on the last day of life.

TABLE VII. MONKEY 79. WEIGHT 2.4 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KILO.) EFFECT OF CYSTEINE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	----	----	0.026	----
1	1700	Tyrosine 4.8 Gm.	2.21	46.0
2	1700	Tyrosine 4.8 Gm.	3.38	70.0
3	1600	Cysteine (CSH) 200 mg.	3.29	68.6
	1700	Tyrosine 4.8 Gm.		
	2200	CSH 200 mg.		
4	0600	CSH 200 mg.	1.49	31.0
	1200	CSH 200 mg.		
	1600	Tyrosine 4.8 Gm.		
	1800	CSH 200 mg.		
	2300	CSH 200 mg.		
5	0700	CSH 200 mg.	0.08	1.7
	1100	CSH 400 mg.		
	1600	CSH 400 mg.		
	1700	Tyrosine 4.8 Gm.		
	2300	CSH 400 mg.		
6	0700	CSH 400 mg.		
	1200	Died		

Monkey 116 had been without ascorbic acid for forty-two days but was not scorbutic when tyrosine loading was commenced. A tyrosyluria did develop on the third day of loading, and injections of glutathione were initiated. But after five days toxic reactions prevented continuance of the experiment. There was a reduction of the tyrosyluria.

Monkey 117 had been without ascorbic acid for forty-two days but was not scorbutic when the loading with tyrosine was commenced. On the third day of loading intramuscular injections of BAL (British anti-lewisite) (2,3-dimercaptopropanol) were commenced. On the first three days two injections of 20 mg. each were given but this caused vomiting. On the fourth day four injections of 10 mg. each were administered but the vomiting still continued. On the following three days, therefore, only three injections of 10 mg. each were used and the vomiting stopped. Table X gives the results of these experiments. When vomiting occurred the results might be too high, depending on whether the load of L-tyrosine had had time to pass out of the stomach.

It has been pointed out by Lugg[34] that the Millon reaction for tyrosine is interfered with by the presence of sulfhydryl compounds in too high concentration. It might be objected that the apparent lowering of tyrosyluria was due to this effect. Accordingly the tyrosyl compounds in a specimen of urine were determined before and after the precipitation of any sulfhydryl compound by Graaf's micro modification of the Hopkins-Vickery procedure.[35] There was no significant change in the amount found.

It appears that sulfhydryl compounds can induce a lowering of the tyrosyluria produced by loading a scorbutic animal with tyrosine, but only when

TABLE VIII. MONKEY 86. WEIGHT 1.45 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KILO.) EFFECT OF CORTICOTROPIN AND CORTISONE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	----	----	0.07	----
1	1800	Tyrosine 2.9 Gm.	2.31	79.5
2	1600	Tyrosine 2.9 Gm.	2.45	84.5
3	1600	ACTH 10 mg.	2.22	76.6
	1700	Tyrosine 2.9 Gm.		
	2300	ACTH 10 mg.		
4	0700	ACTH 10 mg.	1.79	61.8
	1200	ACTH 10 mg.		
	1500	ACTH 10 mg.		
	1600	Tyrosine 2.9 Gm.		
	2200	ACTH 10 mg.		
5	0700	ACTH 10 mg.	1.72	59.3
	1200	ACTH 10 mg.		
	1500	ACTH 10 mg.		
	1600	Tyrosine 2.9 Gm.		
	2200	ACTH 10 mg.		
6	0600	ACTH 10 mg.	1.90	65.6
	1200	ACTH 10 mg.		
	1600	Tyrosine 2.9 Gm.		
	1700	ACTH 30 mg.		
7	0800	ACTH 14.5 mg.	0.66	22.8
	1600	Tyrosine 2.9 Gm.		
8	1600	Tyrosine 2.9 Gm.	1.02	35.3
	1700	Cortisone acetate 25 mg.		
9	0700	Cortisone acetate 25 mg.	0.58	20.0
	1200	Cortisone acetate 25 mg.		
	1600	Cortisone acetate 25 mg.		
	1700	Tyrosine 2.9 Gm.		
10	0700	Cortisone acetate 25 mg.	0.54	18.6
	1200	Cortisone acetate 25 mg.		
	1600	Cortisone acetate 25 mg.		
	1700	Tyrosine 2.9 Gm.		
11	0800	Cortisone acetate 25 mg.	0.32	
	1200	Cortisone acetate 25 mg.		
12	1200	Died		

given in such high doses that toxic reactions occur. It is therefore doubtful whether the sulfhydryl compound acts physiologically or by virtue of its toxic effects.

In another experiment not reported in detail in this paper, 500 mg. of glutathione were injected intramuscularly in a scorbutic animal twice daily for three days before loading with tyrosine was commenced, and continued during loading. The excretion of tyrosyl compounds on the first and second day of loading was only 10.3 and 17.6 per cent of the load. Unfortunately the specimens were lost in the third and fourth days, and the animal died suddenly in the fifth day.

TABLE IX. MONKEY 108. WEIGHT 2.4 KG. ASCORBIC ACID DEFICIENT, LOADED WITH L-TYROSINE (2 GM./KILO.) EFFECT OF CORTICOTROPIN, CORTISONE, FOLINIC ACID AND ASCORBIC ACID ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	----	----	0.038	----
1	1700	Tyrosine 5.0 Gm.	0.93	18.6
2	1700	Tyrosine 5.0 Gm.	1.34	22.7
3	An unknown amount lost during gavage		----	----
4	1700 1800	ACTH 10 mg. Tyrosine 5.0 Gm.	1.86	37.2
5	1500 1745 1800	ACTH 10 mg. ACTH 10 mg. Tyrosine 5.0 Gm.	3.24	64.8
6	1400 1700 1800	ACTH 10 mg. ACTH 10 mg. Tyrosine 5.0 Gm.	2.72	54.4
7	0800 1700 1800	ACTH 10 mg. ACTH 10 mg. Tyrosine 5.0 Gm.	3.31	66.2
8	0900 1700 1800	ACTH 10 mg. ACTH 20 mg. Tyrosine 5.0 Gm.	3.36	67.2
9	1400 1800 1830	ACTH 20 mg. ACTH 20 mg. Tyrosine 5.0 Gm.	2.54	50.8
10-13		No treatment		
14	1500 1700	Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	1.98	39.6
15	1300 1700	Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	2.70	54.2
16	1300 1800	Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	2.84	56.8
17	1700 1730	Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	3.32	66.4
18	0900 1700 1730	Cortisone acetate 25 mg. Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	3.86	77.2
19	0900 1700 1730	Cortisone acetate 25 mg. Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	3.34	66.9
20	0900 1700 1700 1800	Cortisone acetate 25 mg. Cortisone acetate 50 mg. Folinic acid 2 mg. Tyrosine 5.0 Gm.	2.96	59.1
21	1100 1600 1700	Folinic acid 1 mg. Cortisone acetate 50 mg. Tyrosine 5.0 Gm.	2.80	56.0
22	1000 1700 1800	Folinic acid 1 mg. Folinic acid 1 mg. Tyrosine 5.0 Gm.	2.67	53.4

TABLE IX (CONT'D)

23	1100 1630 1700	Folinic acid 1 mg. Folinic acid 1 mg. Tyrosine 5.0 Gm.	1.41	28.2
24	1100 1700 1700	Folinic acid 1 mg. Ascorbic acid 100 mg. Tyrosine 5.0 Gm.	0.96	19.2
25	0900 1700	Ascorbic acid 100 mg. Tyrosine 5.0 Gm.	0.86	17.2
26	1300 1700	Ascorbic acid 100 mg. Tyrosine 5.0 Gm.	0.73	14.4
27	0800 1700	Ascorbic acid 100 mg. Tyrosine 4.6 Gm.	0.81	16.2
28	0800 1700	Ascorbic acid 100 mg. Tyrosine 4.6 Gm.		
37	0800 1700	Ascorbic acid 100 mg. Tyrosine 4.6 Gm.	0.33	7.1
38	0800 1700	Ascorbic acid 100 mg. Tyrosine 4.6 Gm.	0.35	7.9

Adrenal Insufficiency.—Both adrenals were removed from a monkey to see if a spontaneous or induced tyrosyluria would occur in the absence of adrenal hormones; ascorbic acid intake was kept normal. The data from this animal are presented in Table II where it can be seen that tyrosyluria did not occur either spontaneously or after a load of L-tyrosine was administered.

That no adrenal hormones were available from any source in this animal is clear from the following account of its course:

TABLE X. MONKEY 113. WEIGHT 1.68 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KILO.) EFFECT OF FOLINIC ACID AND CORTISONE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
1-7	one/day	Folinic acid 2.0 mg.	----	----
8	1600 1800	Folinic acid 2.0 mg. Tyrosine 3.4 Gm.	1.34	39.4
9	1600 1800	Folinic acid 2.0 mg. Tyrosine 3.4 Gm.	2.42	71.2
10	1900 1800	Folinic acid 2.0 mg. Tyrosine 3.4 Gm.	2.30	67.6
11	1800 1800	Folinic acid 2.0 mg. Tyrosine 3.4 Gm.	2.14	63.0
12	1600 1700	Cortisone acetate 10 mg. Tyrosine 3.4 Gm.	2.18	64.0
13	1600 1700	Cortisone acetate 10 mg. Tyrosine 3.4 Gm.	1.22	35.8
14	1600 1700	Cortisone acetate 10 mg. Tyrosine 3.4 Gm.	Specimen incomplete	
15	1400	Died		

TABLE XI. MONKEY 115. WEIGHT 2.0 KG. RECEIVING 50 MG. L-ASCORBIC ACID PER DAY AND LOADED WITH L-TYROSINE (2 GM./KILO.). EFFECT OF IODOACETATE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
1	1700	Tyrosine 4.0 Gm.	- -	- -
2	1700	Tyrosine 4.0 Gm.	- -	- -
3	1700	Tyrosine 4.0 Gm.	0.42	10.5
4	1600 1600	Tyrosine 4.0 Gm. Iodoacetate 40 mg.	0.60	14.9
5	1600 1600	Tyrosine 4.0 Gm. Iodoacetate 40 mg.	0.27	6.8
6	1600 1700	Iodoacetate 80 mg. Tyrosine 4.0 Gm.	0.37	9.2
7	1600 1600	Iodoacetate 120 mg. Tyrosine 4.0 Gm. Died		

TABLE XII. MONKEY 116. WEIGHT 2.45 KG. ASCORBIC ACID DEFICIENT LOADED WITH L-TYROSINE (2 GM./KILO.). EFFECT OF GLUTATHIONE ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	----	----	0.03	- -
1	0900	Tyrosine 4.9 Gm.	0.55	11.2
2	0900	Tyrosine 4.9 Gm.	0.97	19.7
3	0900 0930 1700	Glutathione (GSH) 500 mg. Tyrosine 4.9 Gm. GSH 500 mg.	1.44	29.4
4	0900 0930 1700	GSH 500 mg. Tyrosine 4.9 Gm. GSH 500 mg.	1.02	20.8
5	0900 0930 1700	GSH 500 mg. Tyrosine 4.9 Gm. GSH 500 mg.	0.90	18.3
6	0830 0900 1700	GSH 500 mg. Tyrosine 4.9 Gm. GSH 500 mg.	0.75	15.4
7	0830 0900	GSH 500 mg. Tyrosine 4.9 Gm.	0.46	9.4

The monkey was maintained on adrenal hormones for the first three post-operative days. Fifty milligrams of ascorbic acid was continued daily. The regimen during experimentation consisted of three feedings a day of the basal milk diet to which NaCl (.8 gram per liter) and $NaHCO_3$ (1.4 grams per liter) were added to provide an additional 50 milliequivalents of Na and 30 milliequivalents of Cl per liter of milk (500 to 600 c.c. were consumed daily).

General vigor was normal, and the weight was stabilized slightly below the preoperative value. The administration of corticotropin seven and eight months after adrenalectomy failed to produce a decline in the level of eosinophils in the peripheral blood or an increase in the urinary excretion of 17-ketosteroids. The monkey was sacrificed eleven months after adrenalectomy, and a thorough postmortem examination failed to disclose gross or microscopic evidence of any accessory adrenal tissue or remnants of either adrenal gland.

Effect of Sulfhydryl Group Inhibitors on the Excretion of p-hydroxyphenyl Compounds.—

(a) *Iodoacetate:* An aqueous solution of sodium iodoacetate was prepared by dissolving 1.6 Gm. of iodoacetic acid in water, carefully neutralizing with dilute caustic soda and making the final volume to 20 ml.

Monkey 115 was a normal healthy young animal. After loading with tyrosine for three days a single injection of iodoacetate solution was given intramuscularly each day (40 to 120 mg.) (Table XI). The final dose proved lethal.

TABLE XIII. MONKEY 117. WEIGHT 2.19 KG. ASCORBIC ACID DEFICIENT, LOADED WITH L-TYROSINE. EFFECT OF BAL (2,3-DIMERCAPTOPROPANOL) ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	--	--	0.02	--
1	0900	Tyrosine 4.4 Gm.	0.34	7.6
2	0900	Tyrosine 4.4 Gm.	1.08	24.5
3	0900	BAL 20 mg.	0.52	11.8
	0930	Tyrosine 4.4 Gm.		
	1600	BAL 20 mg.		
4	0900	BAL 20 mg.	0.15	3.4
	0930	Tyrosine 4.4 Gm.		
	1600	BAL 20 mg.		
5	0800	BAL 20 mg.	0.47	10.7
	0830	Tyrosine 4.4 Gm.		
	1700	BAL 20 mg.		
6	0830	BAL 10 mg.	1.15	26.1
	0900	Tyrosine 4.4 Gm.		
	1200	BAL 10 mg.		
	1600	BAL 10 mg.		
	1900	BAL 10 mg.		
7	0800	BAL 10 mg.	0.99	22.5
	0900	Tyrosine 4.4 Gm.		
	1200	BAL 10 mg.		
	1800	BAL 10 mg.		
8	0800	BAL 10 mg.	0.90	20.4
	1000	Tyrosine 4.4 Gm.		
	1200	BAL 10 mg.		
	1600	BAL 10 mg.		
9	0800	BAL 10 mg.	2.15	48.0
	0900	Tyrosine 4.4 Gm.		
	1200	BAL 10 mg.		
	1600	BAL 10 mg.		
10	0800	BAL 10 mg.	1.65	37.6
	0900	Tyrosine 4.4 Gm.		
11	0900	Tyrosine 4.4 Gm.	1.93	43.9
12	0900	Tyrosine 4.4 Gm.	0.88	20.0
13		----	1.24	28.2

Monkey 33 was a mature female which had been receiving ascorbic acid regularly. After three days of tyrosine loading, injections of iodoacetate were commenced (Table IV). On the fifth day of injections the volume of the urine decreased abruptly and the excretion was bloody. A blood urea nitrogen done that day was not elevated. Injections and loading were continued, and two days later death intervened.

(b) *p-chloromercuri-benzoate:* A solution was prepared by stirring 100 mg. of p-chloromercuri-benzoate with 50 ml. of 0.9 per cent NaCl solution and adding dilute caustic soda drop by drop to pH 7.2.

Monkey 151 was loaded with L-tyrosine at a level of 1.4 Gm. per kilogram of body weight. On the third day of loading 2 mg. of the p-chloromercuri-benzoate were injected intramuscularly. Each day the dose was increased (Table XII). After four days there were still no signs of any effect. On the fifth day a dose of 100 mg. of p-chloromercuri-benzoate was added to the tyrosine gavage. This caused vomiting the following day, so administration of p-chloromercuri-benzoate was stopped for four days. On the ninth day of test it was discovered that ascorbic acid had inadvertently been omitted from the diet of the animal for the past seventeen days. One hundred mg. of ascorbic

TABLE XIV. MONKEY 151. WEIGHT 3.2 KG. RECEIVING ASCORBIC ACID AND LOADED WITH L-TYROSINE (1.3 GM./KILO.). EFFECT OF P-CHLOROMERCURI-BENZOATE (PCMB) ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
0	--	--	0.06	--
1	1600	Tyrosine 4.4 Gm.	0.30	6.8
2	1600	Tyrosine 4.4 Gm.	0.30	6.8
3	1600	PCMB 2 mg. i.m.	0.34	7.8
	1700	Tyrosine 4.4 Gm.		
4	1700	PCMB 4 mg. i.m.	0.52	11.8
	1800	Tyrosine 4.4 Gm.		
5	1700	PCMB 4 mg. i.m.	0.33	7.5
	1800	Tyrosine 4.4 Gm.		
	1900	PCMB 2 mg. i.m.		
6	0900	PCMB 4 mg. i.m.	0.24	5.5
	1700	PCMB 10 mg. i.m.		
	1800	Tyrosine 4.4 Gm.		
7	1000	PCMB 20 mg. i.m.	0.23	5.3
	1700	PCMB 20 mg. i.m.		
	1800	PCMB 100 mg. by gavage		
	1800	Tyrosine 4.4 Gm.		
8	1000	PCMB 20 mg. i.m.	0.33	7.6
	1800	PCMB 8 mg. i.m.		
	1800	Tyrosine 4.4 Gm.		
9	1800	Tyrosine 4.4 Gm.	1.13	25.7
10	1800	Tyrosine 4.4 Gm.	0.73	16.6
11		--	--	--
12	1800	Tyrosine 4.4 Gm.	0.47	10.6
13	1800	PCMB 100 mg. by gavage	0.28	6.4
	1800	Tyrosine 4.4 Gm.		
14	1800	PCMB 100 mg. by gavage	0.95	21.6
	1800	Tyrosine 4.4 Gm.		
15	1800	PCMB 100 mg. by gavage	0.28	6.4
	1800	Tyrosine 4.4 Gm.		
16	1800	PCMB 100 mg. by gavage	0.40	9.0
	1800	Tyrosine 4.4 Gm.		

TABLE XV. MONKEY 164. WEIGHT 1.54 KG. SCORBUTIC, LOADED WITH L-TYROSINE (2 GM./KILO.). EFFECT OF D-ASCORBIC ACID ON THE EXCRETION OF P-HYDROXY-PHENYL COMPOUNDS

DAY OF EXPERIMENT	HOUR	TREATMENT	TOTAL P-HYDROXY-PHENYL COMPOUNDS EXCRETED GM./24 HR.	PER CENT OF LOAD OF TYROSINE EXCRETED AS P-HYDROXY-PHENYL COMPOUNDS
1-12	0900	D-ascorbic acid 50 mg. i.m.	.005	- -
13	0900	D-ascorbic acid 50 mg.	1.45	46.9
	1700	Tyrosine 3.1 Gm.		
14	0900	D-ascorbic acid 50 mg.	2.12	68.4
	1700	Tyrosine 3.1 Gm.		
15-19	0900	D-ascorbic acid 50 mg. i.m.		
20	0900	D-ascorbic acid 50 mg.	1.30	38.3
	1700	Tyrosine 3.1 Gm.		
21	0900	D-ascorbic acid 50 mg.	1.12	32.9
	1700	Tyrosine 3.1 Gm.		

acid were at once given orally and 50 mg. a day thereafter. Loading was then recommenced by giving 100 mg. along with the tyrosine gavage. After four days severe diarrhea set in and the experiment was terminated.

Iodoacetate even in toxic doses does not produce tyrosyluria in a monkey receiving adequate ascorbic acid. The more general sulfhydryl group inhibitor, p-chloromercuri-benzoate, also fails to do this. The rise in the excretion of p-hydroxyphenyl compounds on the ninth day of the loading test on Monkey 151 may have been due to its becoming depleted in ascorbic acid; because on giving the vitamin, the level of excretion of these compounds returned to that of a normal animal under the conditions of the experiment.

The Effect of D-Ascorbic Acid on the Excretion of p-hydroxy-phenyl Compounds by a Scorbutic Monkey Loaded with L-tyrosine.—Monkey 164 was grossly scorbutic when daily injections of 50 mg. of D-ascorbic acid* were commenced. After twelve days a tyrosine loading test was done. Tyrosyluria was still pronounced. Injections of D-ascorbic acid were continued, and a second tyrosine loading test was done a week later. There was still tyrosyluria although somewhat lower (Table XIV). This is in sharp contrast to the prompt effect of L-ascorbic acid.

DISCUSSION

The present report is concerned exclusively with *in vivo* studies of tyrosine metabolism and hence much excellent research by *in vitro* methods has not been mentioned. Work along these lines[36-49] has led to the concept that ascorbic acid is an essential factor in the fission of the aromatic ring whereby 4 fumarylacetoacetic acid is produced subsequent to the deamination of the tyrosine and its conversion to homogentisic acid. So far no role for vitamin B_{12} nor PGA has been found in this chain of reactions. Perhaps this series of reactions merely represents the route by which excess tyrosine is eliminated

*We are greatly indebted to Professor Fred Smith and Dr. J. Kelvin Hamilton of the Department of Agricultural Biochemistry, University of Minnesota, for the D-ascorbic acid which was synthesized by them. The properties of the D-ascorbic acid are mp 192° C. a $^{20}_{D}$ —22° (H_2O).

from the body. There are other series of reactions by which tyrosine is utilized, for example, the conversion to melanin,[50] and the production of epinephrine. It may be in one of these series of reactions that the aberration which results in tyrosyluria in pernicious anemia occurs and in which vitamins of the B complex play a role.

The experiments with the adrenalectomized animal seem to show that the adrenal body has no direct action on the phase of tyrosine metabolism under consideration. The effects produced by cortisone must be through a pharmacological rather than a physiological action. The results with sulfhydryl compounds would seem to indicate that at levels at which they would be effective in reducing the tyrosyluria they are far too toxic to have any physiologic significance in this connection. This is particularly the case with cysteine. The experience of Carey et al.[29] of the toxicity of these compounds in various animals, being higher in larger species, agrees with this. Knox[45] also found that cysteine would not replace L-ascorbic acid in *in vitro* experiments. The experiments with iodoacetate and p-chloromercuri-benzoate show it to be improbable that sulfhydryl activated enzymes are involved in the disposition of p-hydroxy-phenyl compounds.

CONCLUSIONS

Folinic acid had no effect in preventing the excretion of p-hydroxy-phenyl compounds by scorbutic monkeys loaded with L-tyrosine.

Corticotropin was without marked effect on the tyrosyluria produced in a scorbutic monkey.

Cortisone had a variable effect on tyrosyluria in scorbutic monkeys.

An adrenalectomized monkey, which was not lacking ascorbic acid, did not show tyrosyluria when loaded with L-tyrosine.

Cysteine and glutathione, even at toxic levels, did not abolish the tyrosyluria produced in a scorbutic monkey by loading with L-tyrosine.

The sulfhydryl group inhibitors iodoacetate acid and p-chloromercuribenzoate did not cause the development of a tyrosyluria in a monkey supplied with adequate ascorbic acid and loaded with L-tyrosine.

D-ascorbic acid had only a very small and long delayed effect in reducing the tyrosyluria produced in a scorbutic monkey by loading with L-tyrosine.

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THE COMPARATIVE EFFECTS OF MENADIONE SODIUM BISULFITE AND VITAMIN K_1 ON THE HYPOPROTHROMBINEMIA INDUCED BY DICUMAROL*

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THE purpose of this study was to compare the effect of two preparations having vitamin K activity, menadione, and vitamin K_1, on the hypoprothrombinemia induced by Dicumarol and to study further the effectiveness of vitamin K_1 as an antagonist to Dicumarol.

In 1944, Cromer and Barker[1] found that when the prothrombin times of patients receiving Dicumarol became excessively prolonged, the intravenous administration of 64 mg. of menadione sodium bisulfite usually returned the prothrombin times to therapeutic levels. These observations have been confirmed by Overman and his co-workers.[2]

Comparative studies, by James, Bennett, Scheinberg, and Butler[3] and by Miller, Harvey, and Finch,[4] of the action of vitamin K preparations on the hypoprothrombinemia induced by Dicumarol have indicated that vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone) and its oxide are more effective than menadione sodium bisulfite (2-methyl-1,4-naphthoquinone sodium bisulfite) or synkayvite (tetrasodium 2-methyl-1,4-naphthohydroquinone phosphoric acid ester) in counteracting a prothrombin deficiency induced by Dicumarol. These same studies indicated that menadione sodium bisulfite exerts little or no action in correcting the prothrombin deficiency induced by Dicumarol.

The foregoing results concerning the effect of menadione sodium bisulfite as an antagonist to Dicumarol are not in agreement. However, in one instance the menadione was used to correct excessive hypoprothrombinemia which occurred in apparently hypersensitive patients after usual doses of Dicumarol, and in the other instance the menadione was used in an attempt to restore to normal a moderate hypoprothrombinemia in patients with average sensitivity to Dicumarol.

METHODS OF STUDY AND MATERIALS USED

The anticoagulant effect of Dicumarol and the counteracting effect of menadione sodium bisulfite and vitamin K_1 were measured by the one-stage prothrombin time test in all cases. The tests were performed on undiluted plasma by the original method of Quick and co-workers[5] using a system of controls developed for the purpose of reproducibility of results and

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employing a saline extract of dried rabbit brain as the thromboplastic agent.[6, 7] With this method the normal prothrombin time is considered to be 17 to 19 seconds. The initial or control prothrombin time was 17 to 20 seconds in all of the subjects included in this study; this is the usual range for hospitalized patients without hepatic disease or blood dyscrasias who are not receiving anticoagulants.

In this study the term "hypoprothrombinemia" is defined as that coagulation defect indicated by a prolongation of the one-stage prothrombin time, without regard for selective effects which may be measured by the two-stage prothrombin test, or tests for the various accelerators or inhibitors of prothrombin conversion. It is widely recognized that more than prothrombin is reflected by this one-stage test.

The arbitrary therapeutic range of hypoprothrombinemia which is used currently at the Mayo Clinic[8] during treatment with Dicumarol, for the purpose of preventing thrombosis, includes prothrombin times from 27 to 58 seconds. These figures correspond to percentages of prothrombin activity from 30 to 10 per cent of normal. The percentages of prothrombin activity are calculated from a curve based on the results of prothrombin time tests done on various dilutions of normal human plasma, diluted with prothrombin-free human plasma.[7]

Blood for prothrombin time determinations was drawn daily between 8 A.M. and 9 A.M. In one part of the study prothrombin times were determined four and eight hours after the administration of vitamin K_1. Dicumarol was administered orally in the form of compressed tablets, the range of daily dosage varying from 50 mg. to 300 mg. and the entire amount which was given in any one day was given in a single dose. Menadione sodium bisulfite was administered slowly intravenously in an aqueous solution containing 7.2 mg. per milliliter. Menadione sodium bisulfite (250 mg.) was administered orally in the form of capsules. Vitamin K_1 was administered orally in gelatin capsules each of which contained 50 or 250 mg.

The respective routes of administration and doses of these two antagonists to Dicumarol used in the comparative studies were selected for the following reasons. The maximal effective dose of vitamin K_1 given orally for correcting the hypoprothrombinemia induced by Dicumarol has been stated to be 500 mg. However, it has been found that 250 mg. appears to be just as effective in almost all patients. The intravenous administration of 64 mg. of anhydrous menadione sodium bisulfite was employed by Cromer and Barker[1] who felt that such a dosage was effective in converting excessive hypoprothrombinemia to therapeutic hypoprothrombinemia. Studies by James, Bennett, Scheinberg, and Butler[3] suggested that larger doses of menadione sodium bisulfite did not exert any greater antagonistic action on the hypoprothrombinemia induced by Dicumarol. However, the molecular weight of vitamin K_1 is only 1.6 times that of menadione sodium bisulfite; hence 250 mg. of vitamin K_1 on a molecular weight basis is equivalent to 158 mg. of menadione sodium bisulfite. Since the hydrated menadione sodium bisulfite is available in 72 mg. ampules, for purposes of convenience, doses of 144 mg. of menadione bisulfite (2 ampules) and 250 mg. of vitamin K_1 were used in comparing the effect of the two antagonists. They contain approximately equal amounts of methyl naphthoquinone.

OBSERVATIONS

I. *Comparison of Effect of Menadione Sodium Bisulfite and Vitamin K_1 on Similar Degrees of Therapeutic Hypoprothrombinemia Induced by Identical Doses of Dicumarol in the Same Patient.*—Each of 13 patients who had consented to take part in this investigation was studied in the following manner: Dicumarol was given orally in doses of 200 mg. or 300 mg. the first day and 100 mg. or 200 mg. the second day. Subsequent daily doses of Dicumarol (50 to 100 mg.) were given as needed to increase the prothrombin time to approximately 35 seconds. Then administration of Dicumarol was discontinued and the period of time required for the prothrombin time to return to normal was

determined. Recovery was considered complete when the prothrombin time was normal on two consecutive days. This constituted a control study. Then identical doses of Dicumarol were administered on days corresponding to the days of administration in the control study. On the day corresponding to the one when the maximal prothrombin time had been noted in the control study, 144 mg. of menadione sodium bisulfite was administered intravenously. The period of time required for the prothrombin time to return to normal was determined. A similar hypoprothrombinemia was induced for the third time by identical doses of Dicumarol on the same corresponding days. Vitamin K_1

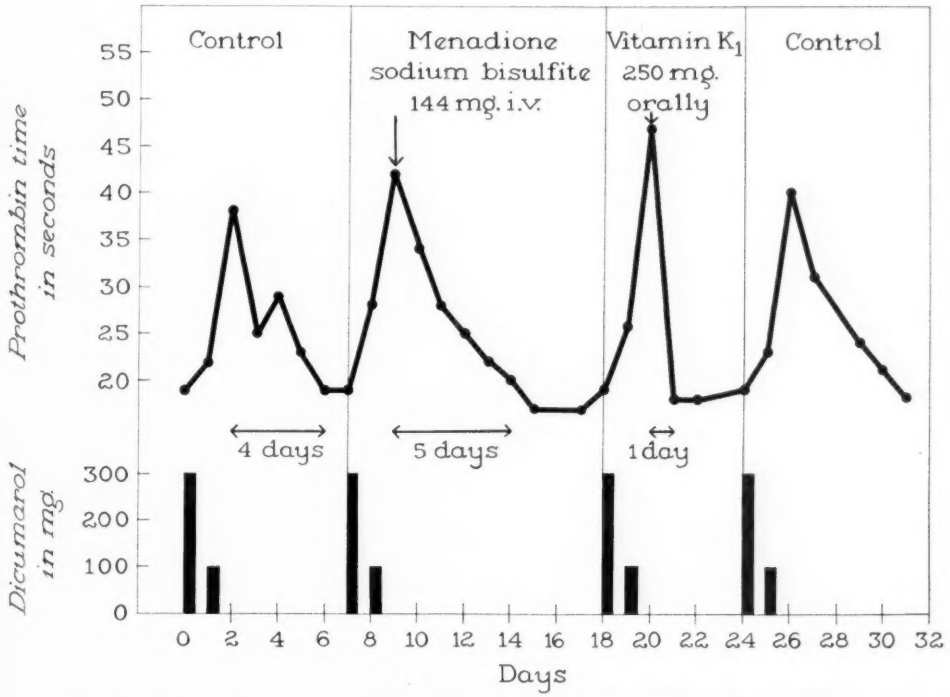

Fig. 1.—A comparison of the effect of 144 mg. of menadione sodium bisulfite intravenously and 250 mg. of vitamin K_1 orally on the hypoprothrombinemia induced by Dicumarol in a representative patient (patient 10). No evidence of refractoriness to reinduction of hypoprothrombinemia was noted after vitamin K_1 was given.

(250 mg.) was administered orally on the day corresponding to the one when the menadione sodium bisulfite had been given and again the time required for the prothrombin time to return to normal was determined.

The period of time required for the spontaneous recovery from the hypoprothrombinemia induced by Dicumarol established a control period against which the effect of menadione sodium bisulfite and vitamin K_1 could be judged in the same patient. Thus, any decrease in the period of time required for recovery, following the administration of each vitamin K antagonist, could be attributed to the effect of the respective antagonist.

TABLE I. COMPARISON OF EFFECT OF MENADIONE SODIUM BISULFITE AND VITAMIN K_1 ON THERAPEUTIC HYPOPROTHROMBINEMIA INDUCED BY DICUMAROL IN THE SAME PATIENT

PATIENT	DURATION OF PERIOD OF DICUMAROL ADMINISTRATION DURING EACH STUDY PERIOD, DAYS	AMOUNT OF DICUMAROL ADMINISTERED DURING EACH STUDY PERIOD, MG.	MAXIMAL PROTHROMBIN TIME, SEC.			TIME IN DAYS REQUIRED FOR THE PROTHROMBIN TIME TO RETURN TO NORMAL		
			CONTROL PERIOD	BEFORE MENADIONE SODIUM BISULFITE	BEFORE K_1	CONTROL PERIOD	AFTER 144 MG. OF MENADIONE SODIUM BISULFITE INTRAVENOUSLY	AFTER 250 MG. OF VITAMIN K_1 ORALLY
1	3	500	37	40	46	3	6	1
2	4	700	32	32	38	6	6	1
3	2	500	36	28	36	5	4	1
4	3	600	31	39	31	4	6	1
5	3	600	34	34	39	5	4	2
6	2	500	39	60	37	7	4	2
7	2	500	41	40	52	7	9	4
8	2	500	46	47	63	10	10	4*
9	4	550	30	36	38	3	3	1
10	2	400	38	42	47	4	5	1
11	2	400	35	49	39	4	5	1
12	2	400	35	49	38	2	4	1
13	4	700	43	35	41	3	2	1
Average			36.7	40.8	41.9	4.8	5.2	1.6

*Patient vomited after vitamin K_1 was given.

The results obtained are shown in Table I and illustrated in Fig. 1: menadione sodium bisulfite did not decrease the recovery period over that of the control period. Vitamin K_1 reduced the recovery period over that of the control period by 1 to 6 days, with an average of 3.2 days.

The average time in the control series for the hypoprothrombinemia to return to normal was 4.8 days; following administration of menadione sodium bisulfite the average time was 5.2 days as compared to 1.6 days following administration of vitamin K_1.

II. *Comparison of Effect of Menadione Sodium Bisulfite and Vitamin K_1 in Correcting Marked Hypoprothrombinemia Induced by Dicumarol in Paired Patients.*—The 16 patients included in this study were all hypersensitive to the usual induction doses of Dicumarol, and vitamin K preparations were given to correct the excessive hypoprothrombinemia which had been produced.

The patients were paired as to the amount of Dicumarol given, the length of time after the first dose of Dicumarol when the antagonist was given and the degree of hypoprothrombinemia encountered. In each pair one patient received 72 mg. of menadione sodium bisulfite intravenously and the other patient received 50 mg. of vitamin K_1 orally. On a molecular basis 72 mg. of menadione sodium bisulfite is equivalent to 113 mg. of vitamin K_1; hence the dose of vitamin K_1 was actually equivalent to less than half that of the menadione sodium bisulfite.

The results obtained are shown in Table II. The prothrombin time was decreased in all 8 patients 16 to 20 hours following the intravenous administration of 72 mg. of menadione sodium bisulfite. The range of this drop in the prothrombin time expressed as increase in per cent of normal prothrombin activity varied from 3 per cent to 26 per cent. The average increase was 14.2 per cent.

The prothrombin time was likewise decreased in all 8 patients who received vitamin K_1, and in 7 of them the increase of per cent of normal prothrombin activity was greater than in the corresponding paired patient who received menadione sodium bisulfite. The range of increase in per cent of normal prothrombin activity following oral administration of 50 mg. of vitamin K_1 varied from 7 per cent to 70 per cent (average 39.1 per cent).

III. *The Effectiveness of 250 mg. of Oral Vitamin K_1 in Correcting Varying Degrees of Hypoprothrombinemia Induced by Varying Amounts of Dicumarol Given for Varying Periods in Different Patients.*—This study was made in 22 patients. Dicumarol was employed in 17 as a prophylactic measure following surgical procedures, in 3 because of a recent myocardial infarction, and in 2 because of thrombophlebitis of the veins of the calf.

Of these 22 patients 7 received vitamin K_1 for correction of their hypoprothrombinemia just prior to their dismissal from the hospital, 6 received vitamin K_1 because of a proposed surgical procedure, 5 because of minor bleeding, and 4 because of unusual sensitivity to Dicumarol. Vitamin K_1 was administered four to eight hours after the last dose of Dicumarol.

TABLE II. COMPARATIVE EFFECTS OF 50 MG. OF VITAMIN K_1 ORALLY AND 72 MG. OF MENADIONE SODIUM BISULFITE INTRAVENOUSLY ON COMPARABLE DEGREES OF EXCESSIVE HYPOPROTHROMBINEMIA PRODUCED BY IDENTICAL DOSES OF DICUMAROL IN PAIRED PATIENTS

PAIRED PATIENTS	AMOUNT OF DICUMAROL GIVEN, MG.	TOTAL DAYS OF DICUMAROL THERAPY	ANTAGONIST WAS GIVEN NORMAL PROTHROMBIN BEFORE ONE-STAGE PROTHROMBIN TIME IN SECONDS AND PER CENT OF		ONE-STAGE PROTHROMBIN TIME IN SECONDS AND IN PER CENT OF NORMAL PROTHROMBIN ACTIVITY 16 TO 20 HOURS AFTER ANTAGONIST WAS GIVEN*			
					VITAMIN K_1, 50 MG. ORALLY		MENADIONE SODIUM BISULFITE, 72 MG. INTRAVENOUSLY	
			SECONDS	% OF NORMAL PROTHROMBIN ACTIVITY	SECONDS	% OF NORMAL PROTHROMBIN ACTIVITY	SECONDS	% OF NORMAL PROTHROMBIN ACTIVITY
14	300	12	96	7	25	40	30	26.5
15	300	12	108	5				
16	300	12	58	10	19	80	31	25
17	300	12	62	10				
18	300	12	75	9	22	54	26	35
19	300	12	78	9				
20	300	12	56	11	26	35	33	21
21	300	12	67	9				
22	300	12	83	8	24	45	45	14
23	300	12	92	7				
24	450	12	57	10	21	60	33	21
25	450	12	67	9				
26	300	12	67	9	40	16	29	27
27	300	12	76	9				
28	300	12	91	7	22	54	52	11
29	300	12	86	8				

*In each case the antagonist was given approximately 24 hours after the last dose of Dicumarol had been given.

The results obtained are shown in Table III. The prothrombin time just before administration of vitamin K_1 in the 22 patients averaged 54 seconds, the range varying from 27 seconds to 132 seconds. The prothrombin time 16 to 20 hours after administration of vitamin K_1 (250 mg.) averaged 21 seconds, the range varying from 17 seconds to 30 seconds. Sixteen to 20 hours following the administration of vitamin K_1 16 patients had essentially normal prothrombin times (17 to 21 seconds); 3 patients had a prothrombin time of 22 seconds, and 1 patient had a prothrombin time of 23 seconds. Two patients had prothrombin times of 26 seconds and 30 seconds, respectively.

TABLE III. THE EFFECTIVENESS OF 250 MG. OF VITAMIN K_1 ORALLY IN CORRECTING VARYING DEGREES OF HYPOPROTHROMBINEMIA INDUCED BY VARYING AMOUNTS OF DICUMAROL GIVEN FOR VARYING PERIODS IN DIFFERENT PATIENTS

PATIENT	AMOUNT OF DICUMAROL GIVEN, MG.	TOTAL DAYS OF DICUMAROL THERAPY	PROTHROMBIN TIME IN SECONDS BEFORE 250 MG. OF ORAL VITAMIN K_1	PROTHROMBIN TIME IN SECONDS 16 TO 20 HOURS AFTER 250 MG. OF ORAL VITAMIN K_1
30	2,525	33	48	21
31	625	10	27	22
32	350	3	29	19
33	625	8	33	22
34	1,400	12	45	21
35	1,000	12	70	30
36	800	9	41	23
37	425	4	34	20
38	2,500	44	44	20
39	475	5	40	21
40	625	8	32	21
41	1,125	16	30	21
42	475	5	27	20
43	700	5	67	21
44	2,100	25	39	26
45	300	2	108	20
46	325	3	132	22
47	300	2	91	21
48	300	2	36	21
49	500	2	98	17
50	850	5	81	20
51	550	6	33	19
Average			54	21

IV. *The Effectiveness of 250 mg. of Menadione Sodium Bisulfite Given Orally in Correcting Varying Degrees of Hypoprothrombinemia Induced by Varying Amounts of Dicumarol Given for Varying Periods in Different Patients.*—Eighteen patients were included in this study. Dicumarol was employed in 14 as a prophylactic measure following surgical procedures, in 3 patients because of a recent myocardial infarction, and in 1 patient because of thrombophlebitis.

Of these 18 patients 14 received 250 mg. of menadione sodium bisulfite orally because the hypoprothrombinemia was in excess of the therapeutic range, 3 received menadione sodium bisulfite because of minor bleeding, and 1 received it prior to dismissal from the hospital. Menadione sodium bisulfite was administered orally 4 to 8 hours after the last dose of Dicumarol.

The results obtained are shown in Table IV. The prothrombin times prior to administration of menadione sodium bisulfite in the 18 patients averaged 62.8 seconds, the range varying from 31 seconds to 91 seconds. The prothrombin

TABLE IV. THE EFFECTIVENESS OF 250 MG. OF MENADIONE SODIUM BISULFITE ORALLY IN CORRECTING VARYING DEGREES OF HYPOPROTHROMBINEMIA INDUCED BY VARYING AMOUNTS OF DICUMAROL DURING VARYING PERIODS IN DIFFERENT PATIENTS

PATIENT	AMOUNT OF DICUMAROL GIVEN, MG.	TOTAL DAYS OF DICUMAROL THERAPY	PROTHROMBIN TIME IN SECONDS BEFORE 250 MG. OF MENADIONE SODIUM BISULFITE	PROTHROMBIN TIME IN SECONDS 16 TO 20 HOURS AFTER 250 MG. OF MENADIONE SODIUM BISULFITE
52	425	3	65	23
53	300	2	91	38
54	650	5	36	23
55	500	2	60	25
56	300	2	65	22
57	700	8	61	25
58	475	4	42	31
59	500	3	63	26
60	400	2	75	30
61	625	4	67	29
62	500	2	48	25
63	650	4	31	23
64	725	6	55	29
65	625	7	77	22
66	1,450	20	69	35
67	500	2	63	27
68	600	3	78	28
69	550	4	85	23
Average			62.8	26.9

times, 16 to 20 hours after administration of menadione sodium bisulfite, averaged 26.9 seconds, the range varying from 22 seconds to 38 seconds. Following administration of 250 mg. of menadione sodium bisulfite 8 patients had prothrombin times within the therapeutic range (from 27 seconds to 38 seconds). They were below the lower limit of the therapeutic range (26 seconds or below) in the remaining 10 patients.

V. *The Effectiveness and Rapidity of Action of Vitamin K_1 (250 or 500 mg.) Orally on Moderate to Excessive Hypoprothrombinemia After Prolonged Administration of Dicumarol.*—Six patients were studied; all had received prolonged Dicumarol therapy (16 to 57 days) for either acute myocardial infarction or thrombophlebitis involving the lower extremities.

When each patient was ready for dismissal from the hospital and Dicumarol therapy was to be discontinued, the patient was given increased doses of Dicumarol sufficient to increase the prothrombin time to near the upper limit of the

TABLE V. THE EFFECTIVENESS AND RAPIDITY OF ACTION OF VITAMIN K_1 (250 OR 500 MG.) ORALLY ON MODERATE TO EXCESSIVE HYPOPROTHROMBINEMIA AFTER PROLONGED ADMINISTRATION OF DICUMAROL

PATIENT	TOTAL AMOUNT OF DICUMAROL GIVEN, MG.	NO. OF DAYS DICUMAROL WAS EMPLOYED	AMOUNT OF VITAMIN K_1 GIVEN, MG.	PROTHROMBIN TIME IN SECONDS			
				BEFORE VITAMIN K_1	AFTER VITAMIN K_1		
					4 HOURS	8 HOURS	24 HOURS
70	2,575	29	250	69	53	31	20
71	2,275	23	250	46	40	23	20
72	2,200	17	250	55	53	24	20
73	3,450	57	250	47	39	24	19
74	1,250	16	500	60	51	35	21
75	1,575	23	500	50	28	23	21

therapeutic range. Then vitamin K_1 (250 or 500 mg.) was administered orally 16 to 20 hours following the last dose of Dicumarol. Prothrombin times were determined 4, 8, and 24 hours following the administration of vitamin K_1.

The results obtained are shown in Table V. In all 6 patients a slight decrease in prothrombin time was noted 4 hours after administration of vitamin K_1. A significant decrease was noted after 8 hours, and at the end of 24 hours the prothrombin times of all 6 patients were within 1 second of the normal range for hospital patients.

VI. *Comparison of the Maximal Degree of Hypoprothrombinemia Which Occurred During the Initial Control Period and During a Subsequent Study Period Following Oral Administration of 250 mg. of Vitamin K_1. Identical Doses of Dicumarol Were Given on Corresponding Days to Each Patient During Each Period of Study.*—Seven of the 13 patients included in Table I received identical doses of Dicumarol the fourth time, 48 to 120 hours following the oral administration of 250 mg. of vitamin K_1. The maximal degrees of hypoprothrombinemia which occurred during the initial control period and following vitamin K_1 were compared; thus the blocking effect of vitamin K_1 on reinduction of hypoprothrombinemia could be evaluated.

The results of these studies are seen in Table VI and illustrated in Fig. 1. All patients attained a therapeutic hypoprothrombinemia (28 to 46 seconds) with an identical amount of Dicumarol following 250 mg. of oral vitamin K_1. During the period of study 3 of the 7 patients had a greater lengthening of the prothrombin time following administration of vitamin K_1 than before its administration.

In the present study attention has been focused on skeletal muscle ob- the control periods preceding and following the administration of vitamin K_1 were 36.4 and 34.4 seconds, respectively.

COMMENT

It would seem that the best way of comparing the effectiveness of two preparations having vitamin K activity in correcting the hypoprothrombinemia produced by Dicumarol is the procedure summarized in Table I where the comparisons are made in the same patient. In this study vitamin K_1 given orally was found to be consistently effective and menadione sodium bisulfite given intravenously in a comparable molecular dose was found to be consistently ineffective.

However, none of these patients were hypersensitive to Dicumarol, and in considering the clinical needs for antagonists it is obvious that these will frequently arise in hypersensitive patients. It is difficult to make comparisons of two antagonists in hypersensitive patients because after administration of one of them the patient is usually no longer hypersensitive. Even though control studies are lacking, the results in Table II in a series of paired hypersensitive patients at least suggest that vitamin K_1 is a more effective antagonist than menadione sodium bisulfite even when the dose of the former is less than half as great on a molecular basis, and actually smaller in milligrams.

TABLE VI. COMPARISON OF RAPIDITY OF INDUCTION AND DEGREE OF HYPOPROTHROMBINEMIA INDUCED BY DICUMAROL BEFORE AND AFTER ORAL ADMINISTRATION OF 250 MG. OF VITAMIN K_1 IN THE SAME PATIENT

	CONTROL STUDY				AFTER VITAMIN K_1				
PATIENT	CONTROL PROTHROMBIN TIME, SECONDS	AMOUNT OF DICUMAROL GIVEN, MG.	NO. OF DAYS OF DICUMAROL THERAPY	PROTHROMBIN TIME AFTER DICUMAROL, SECONDS	DAYS AFTER VITAMIN K_1 WHEN DICUMAROL RESTARTED	PROTHROMBIN TIME BEFORE DICUMAROL RESTARTED, SECONDS	AMOUNT OF DICUMAROL GIVEN, MG.	NO. OF DAYS OF DICUMAROL THERAPY	PROTHROMBIN TIME AFTER DICUMAROL, SECONDS
1	19	500	3	37	2	18	500	3	46
2	18	700	4	32	4	19	700	4	39
4	20	600	3	36	4	20	600	3	29
5	20	600	3	34	5	19	600	3	29
10	19	400	2	38	4	19	400	2	40
12	20	400	2	35	2	19	400	2	30
13	18	700	4	43	3	19	700	4	28
Average				36.4					34.4

Another comparison may be made between the results which are shown in Tables III and IV. The doses of vitamin K_1 and menadione sodium bisulfite were the same (250 mg.) and were given by the same route (orally). On a molecular basis the dose of menadione sodium bisulfite was larger, but its effect was less consistent and almost always less complete than the effect of vitamin K_1. However, within 24 hours after the administration of menadione sodium bisulfite the prothrombin time was always shorter than before its administration, and sometimes approached normal. We doubt very much that this would have occurred if menadione sodium bisulfite had not been given. It is possible that 72 mg. or even 144 mg. is not the maximal effective dose of menadione sodium bisulfite, and it is possible that this compound is more effective when it is given orally, since it is known that it is excreted rapidly by the kidneys when given intravenously in aqueous solution.

Our studies indicate that vitamin K_1 given orally in a single 250 mg. dose is very consistently effective in correcting within 24 hours the hypoprothrombinemia produced by Dicumarol regardless of the duration of anticoagulant therapy or the amount of Dicumarol which was given.

In 31 of 39 patients who received this dose the prothrombin time was normal or within 1 second of the upper limit of normal for hospital patients within 24 hours. In 4 others it was within a few seconds of normal. In only 4 was the response incomplete and 1 of these vomited soon after the vitamin K had been given. None of these 39 patients had extreme degrees of hypoprothrombinemia.

Refractoriness to coumarin compounds after the administration of 500 mg. of vitamin K_1 has been reported.[4, 9] It is noteworthy that in our controlled study there was no refractoriness to Dicumarol after 250 mg. of vitamin K_1 in the 7 patients who were tested.

Our results in this study support the thesis that vitamin K_1 is the more effective antagonist[10] for completely correcting the hypoprothrombinemia produced by Dicumarol as rapidly as possible but the menadione sodium bisulfite is preferable for bringing the prothrombin time back into the therapeutic range but not below it in a patient who has reacted excessively to the usual induction doses of Dicumarol.

SUMMARY

In this study vitamin K_1 was found to be definitely more effective than menadione sodium bisulfite in correcting the hypoprothrombinemia produced by Dicumarol. Two hundred fifty milligrams of vitamin K_1 given orally almost always restored the prothrombin time to normal within 24 hours regardless of the height of the prothrombin time or the duration of Dicumarol therapy when the vitamin K_1 was given. No refractoriness to reinduction of therapeutic hypoprothrombinemia by Dicumarol was noted after oral administration of 250 mg. of vitamin K_1.

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THE METABOLISM OF WATER AND ELECTROLYTES IN CONGESTIVE HEART FAILURE

II. The Distribution of Water and Electrolytes in Skeletal Muscle in Edematous Patients With Congestive Heart Failure Before and After Treatment

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IN A previous work the distribution of water and electrolytes in normal human skeletal muscle was described.[1] Data for the normal organism having been established, it was desirable to investigate the water and electrolyte changes in edematous skeletal muscle of individuals with congestive heart failure and then to determine the effects of therapy upon these abnormalities.

METHODS

The procedures and methods utilized in muscle biopsy, tissue, and serum analyses, and in calculating the distribution of water and electrolytes in skeletal muscle have been reported previously.[1] In the edematous subjects all biopsies were obtained from the mid thigh and were carried out under local procaine block anesthesia because the severity of the patients' symptoms precluded the use of general anesthesia. The infiltrate of procaine was kept at least 10 cm. from the site of the biopsy, and no procaine was injected into the muscle itself. After exposure of the muscle the sample for analysis was rapidly dissected away with scissors because of the painful nature of the procedure. Once the sample was obtained, the muscle and surrounding connective tissues were widely infiltrated with procaine so as to control the pain. All patients undergoing muscle biopsy were premedicated with 50 to 100 mg. of Demerol. Because of the high venous pressure usually encountered in the patients with severe congestive failure and the resultant tendency to hematoma formation, it was found helpful to place a piece of Gelfoam in the site of the muscle biopsy to act as a hemostatic agent. Samples of muscle obtained after partial or complete relief of edema were taken from the inferior portion of the rectus abdominus muscle or from the mid thigh and were obtained under local, regional, or general anesthesia.

RESULTS

Tables I and III present the original chemical data obtained on serum and skeletal muscle from patients with heart disease. Table I comprises the patients having massive peripheral edema, while Table III comprises the patients who were previously edematous but who were partially or completely free of edema at the time of the biopsy. From the data recorded in Tables I and III, the derived data given in Tables II, IV, and V were calculated. It will be noted that in three instances the tissue analyses reported in Tables III and IV were obtained in patients also reported in Tables I and II.

Distribution of Tissue Water.—In the patients with massive peripheral edema (indicated as four plus in Table I) the total water content is con-

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TABLE I. ANALYSES OF SERUM AND TISSUES IN EDEMATOUS SUBJECTS

PATIENT AGE	SITE		pH	CO_2 (mM/L.)	H_2O* (GM.)	Cl^{-1} (MEQ.)	Na^{+1} (MEQ.)	K^{+1} (MEQ.)	N* (GM.)	FAT (%)
E. L.$_1$	Quad.	S†	7.35	28.1	917.1	94.5	135.3	4.78		
42	Fem.	M‡			838.3	25.1	30.1	66.40	22.5	0.8
J. R.	Quad.	S	7.39	27.8	910.9	96.0	140.6	4.78		
68	Fem.	M			830.4	24.2	31.0	78.40	23.0	0.8
W. H.	Quad.	S	7.46	32.1	933.5	83.9	123.7	3.91		
76	Fem.	M			801.1	21.0	28.8	96.40	29.4	0.8
C. J.	Quad.	S	7.40	38.1	927.9	80.8	127.4	4.11		
48	Fem.	M			807.5	16.2	29.9	87.70	25.1	5.1
E. L.$_2$	Quad.	S	7.34	16.3	909.5	80.1	110.0	6.48		
42	Fem.	M			831.4	28.2	33.6	74.90	25.8	1.6
M. B.$_1$	Quad.	S	7.48	33.1	917.3	94.7	136.8	4.32		
59	Fem.	M			820.9	31.4	58.3	74.20	27.2	2.1
L. O.	Quad.	S	7.40	24.5	904.4	103.6	136.8	4.12		
54	Fem.	M			804.8	28.1	42.0	85.80	30.9	1.1
S. S.	Quad.	S	7.25	24.2	906.4	87.2	131.7	5.17		
56	Fem.	M			825.6	28.6	31.4	77.40	24.2	0.8
Mean		S	7.38	28.1	915.9	90.1	130.3	4.78		
S.D.§			0.07	6.6	9.6	7.8	9.2	0.78		
Mean		M			820.0	25.4	35.6	80.20	26.0	
S.D.					13.0	4.6	9.4	8.77	2.8	
Controls:										
Mean		S	7.41	26.4	910.8	100.0	136.5	4.34		
S.D.			0.04	2.8	4.6	3.0	2.5	0.31		
Mean		M			776.6	19.1	33.7	94.00	29.5	
S.D.					6.4	3.9	6.4	5.90	2.2	

*Expressed per kilogram of fresh fat-free tissue.
†Serum.
‡Muscle.
§Standard deviation.

DIAGNOSES AND REMARKS (TABLE I)

E. L.$_1$ Rheumatic Heart Disease.—Mitral stenosis and insufficiency, aortic stenosis and insufficiency, tricuspid insufficiency. Chronic congestive failure of ten years' duration. Four plus pitting edema at time of biopsy. Patient on digitalis. Had previously received mercurials and acidifying salts, but none for two weeks prior to biopsy.

J. R. Rheumatic Heart Disease.—Mitral stenosis and insufficiency, chronic congestive heart failure of fifteen years' duration. Four plus edema at time of biopsy. Patient on digitalis. Had received mercurials and acidifying salts previously, but none for two weeks prior to biopsy.

W. H. Arteriosclerotic Heart Disease.—Carcinoma of the prostate with metastases. Congestive heart failure of six months' duration. Four plus edema at time of biopsy. Patient had hyponatremia and had received infusions of hypertonic saline for several days prior to biopsy. Patient on digitalis and had received a mercurial diuretic two days before the biopsy.

C. J. Chronic Cor Pulmonale.—Congestive heart failure of three years' duration. Four plus pitting edema at time of biopsy. Patient had persistent hypochloremic alkalosis. Patient on digitalis, and had previously received mercurials and acidifying salts, but no mercurials for two weeks prior to biopsy.

E. L.$_2$ See E. L.$_1$ above.—This specimen obtained fifteen minutes after death. Blood drawn for serum analyses just before death. Patient had four plus pitting edema at time of death.

H. B.$_1$ Arteriosclerotic Cardiovascular Disease.—Congestive heart failure with edema of six months' duration. Three plus edema at time of biopsy. Patient on digitalis. Had received no mercurials or other diuretics before biopsy.

L. O. Chronic Constrictive Pericarditis.—Edema of eight months' duration. Four plus edema at time biopsy was taken. Patient on digitalis. No previous mercurials.

S. S.$_1$ Rheumatic Heart Disease.—Chronic congestive failure with edema and ascites. Heart failure of 10 years' duration. Four plus edema at time biopsy was obtained. Patient on digitalis and had received many diuretics previously, but none for two weeks prior to the biopsy.

sistently higher than that of normal skeletal muscle.[1] The average total water for the controls was 77.6 ± 0.6 per cent, while the average obtained for edematous patients was 82.0 ± 1.3 per cent. This increase in water content is the result of a significant increase in the extracellular water content and also an increase in the intracellular water as illustrated in Figs. 1 and 2. When the relative values for intracellular water, expressed on the basis of fresh fat-free tissue, are compared, the mean value for the edematous patients [$(H_2O)c$ =

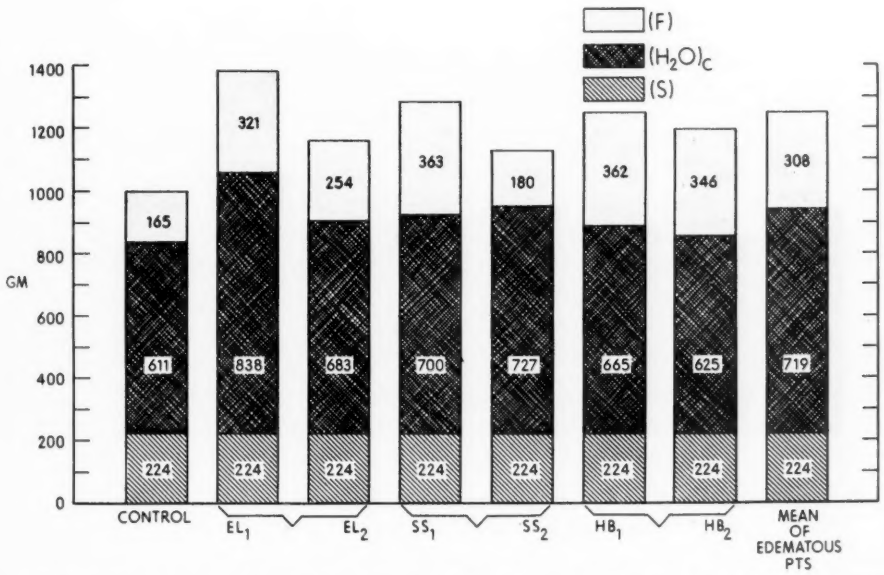

Fig. 1.—Extracellular phase (F), intracellular water $(H_2O)c$, and solids (S) in control subjects,[1] in edematous patients, and in patients following treatment. The first column at the extreme left represents the mean values observed in the control subjects.[1] The next two columns represent samples of muscle obtained from the same subject: $E.L._1$ was obtained at a time when the patient was in severe congestive failure with marked peripheral edema; $E.L._2$ was obtained after the loss of 11.8 kilograms of edema fluid. In the next two columns are represented the values obtained in patient H.B. before and after losing 6 kilograms of edema fluid. The last two columns represent the values for patient S.S. before and after losing 16.4 kilograms of edema. ΔM represents the absolute change in one kilogram of fresh fat-free muscle as referred to the control values[1]; $\Delta(F)$ and $\Delta(H_2O)c$ represent the absolute change in the extra- and intracellular fluid volume per kilogram of fresh fat-free tissue as referred to the controls.[1] Note that in each instance the values for (F) and $(H_2O)c$ are derived by correcting the values recorded in Tables II and IV to a constant value for fat-free tissue solids (S) represented by the mean of the control observations.[1]

575.4 ± 36.5] is not significantly different from that of the controls [$(H_2O)c = 611.0 \pm 30.8$] (Table II). If the intracellular water is calculated in terms of the tissue solids (Table V) (absolute values) it will be noted that the mean value for intracellular water in the edematous subjects [$(H_2O)c = 719 \pm 66$] is significantly greater than the control value [$(H_2O)c = 611.0 \pm 30.8$]. Similarly, the percentage of water in the intracellular phase is significantly greater in the edematous patients [$(H_2O)c = 76.5 \pm 1.5$ per cent] than in the controls [$(H_2O)c = 73.3 \pm 0.1$ per cent].

TABLE II. DERIVED DATA IN EDEMATOUS PATIENTS

PATIENT	$(H_2O)F$ GM./KG. FRESH FAT-FREE TISSUE	$(H_2O)C$ GM./KG. FRESH TISSUE	$\{H_2O\}C$ GM./KG. CELLS	(NA) MEQ./KG. INTRACELLULAR WATER	(K)	(NA + K)	RATIO: (NA)M* / (CL)M*	RATIO: (K)M* / (N)M†
E. $L._1$	231.6	606.7	791.9	0.0	107.5	107.5	1.20	2.96
J. R.	218.2	612.2	785.3	0.0	126.3	126.3	1.28	3.40
W. H.	221.9	579.2	746.5	2.6	166.4	169.0	1.37	3.34
C. J.	176.6	630.9	767.9	10.9	137.9	148.8	1.85	3.47
E. $L._3$	303.9	527.5	761.2	0.0	138.0	138.0	1.19	2.90
H. $B._1$	289.2	531.7	751.1	32.5	141.4	173.3	1.90	2.87
L. O.	233.1	571.7	747.8	14.9	148.3	163.2	1.49	2.76
S. $S._1$	282.4	543.2	760.1	0.0	139.7	139.7	1.10	3.19
Mean	244.6	575.4	764.8	7.6	138.2	145.5	1.42	3.11
S.D.	40.4	36.5	14.6	10.8	15.8	20.8	0.28	0.25
Controls:								
Mean	165.5	611.0	732.8	16.9	152.7	169.6	1.78	3.20
S.D.	33.3	30.8	9.6	7.6	7.9	6.2	0.30	0.30

*Meq./100 Gm. dry fat-free solids.
†Gm./100 Gm. dry fat-free solids.

Following the partial relief of edema in patients $E.L._2$, $H.B._2$, and $S.S._2$ in Tables III and IV, there is noted a reduction in total water content of the tissues, $E.L._2$ decreasing from 83.8 per cent to 80.7 per cent, $H.B._2$ from 82.1 per cent to 81.3 per cent, and $S.S._2$ from 82.6 per cent to 80.2 per cent. If the relative values for extra- and intracellular water after treatment (Table IV) are compared with the relative values before treatment (Table II), extracellular water was decreased significantly in only one subject ($S.S._2$), while intracellular water was increased in the same patient. If the tissue phases of each subject before and after treatment are calculated in terms of the muscle solids (absolute changes) it will be noted that there was a decrease in intracellular water in patient $E.L._2$ but an increase in $S.S._2$ (Fig. 1). Patient $H.B._2$ showed no significant response to treatment.

The values for the distribution of water in the skeletal muscle of patients who had been edematous at one time but who were completely free of edema at the time the biopsy was obtained are listed in Table IV. (Patients J.D., J.M., G.K., W.K., and H.H.). The relative values for intra- and extracellular water obtained in these patients correspond closely to those of the controls.[1] Calculation of the phase mass data in these patients demonstrated the following mean values: (M) = 1042, (F) = 170, $(H_2O)c$ = 648, (S) = 224, and $(H_2O)c$ = 74.4 (see Fig. 3, *C*).

Potassium and Nitrogen.—The tissue potassium content referred to fresh fat-free muscle is decreased in the presence of edema as compared with the controls.[1] This basis of reference, however, is not reliable in the presence of edema since a proportionately greater percentage of the tissue is made up of water. When the tissue potassium is referred to nitrogen, the K:N ratio in edematous cardiacs (3.11 ± 0.25) is slightly lower than in the controls (3.20 ± 0.29). This difference, however, is not statistically significant. The tissue potassium referred to fat-free solids was actually higher in the edematous patients observed (mean value = 44.7 ± 2.00) than in the controls (42.0 ± 2.58 meq./100 Gm. fat-free solids). Following treatment the K:N ratio and the

tissue potassium content expressed on the basis of fat-free solids were not significantly different from the control values.

Sodium and Chloride.—Both the tissue sodium and chloride content were increased in the patients with edema (Table I). These increases are statistically significant and apply when referred to fresh fat-free tissue as well as to fat-free solids. The decreased Na:Cl ratio (Table II) resulted from a greater increase in the tissue chloride than sodium in the patients with cardiac edema. In the treated patients these values are not significantly different from the controls (Tables III and IV).

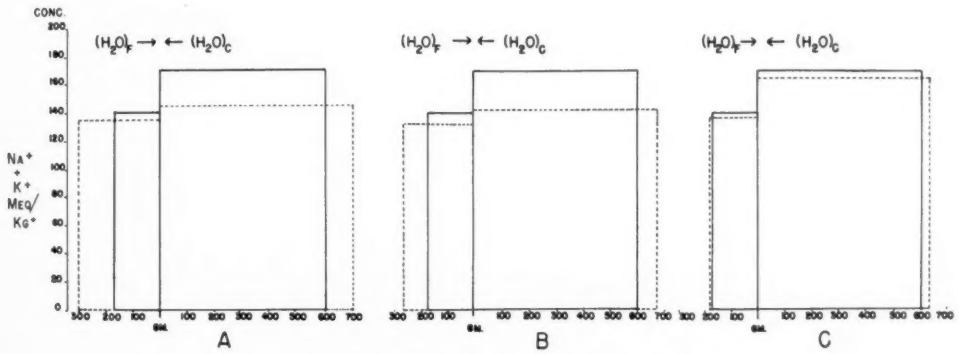

Fig. 2.—Extracellular water $(H_2O)f$ and intracellular water $(H_2O)c$ are plotted along the abscissas; the zero point represents the interface between the two in each case. The sums of the concentrations of the cations sodium and potassium in extra- and intracellular water are plotted along the ordinates. In each figure the solid lines represent the mean values observed in the controls.[1] In "A" the dotted lines represent the mean values observed in the edematous patients in Tables I and II. In "B" the dotted lines represent the mean values observed in the treated patients who were partially free of edema (Table IV). In "C" the dotted lines represent the mean values observed in patients who had been previously edematous, but who at the time of the biopsy were entirely free of edema.

Intracellular Sodium and Potassium.—In the edematous patients studied the mean value for the sum of intracellular sodium and potassium concentration (Table II) is lower than that of the controls.[1] It will be noted that in three of the edematous cardiacs reported in Table II (H.B.$_1$ W.H., and L.O.) the concentration of intracellular sodium plus potassium is not significantly different from the control values.[1] Following treatment and the partial relief of edema (Tables III and IV) the intracellular cation concentration was increased in E.L.$_2$, essentially unchanged in H.B.$_2$,* and decreased in S.S.$_2$ The cardiac patients who were completely free of edema at the time the biopsy was obtained reflected an essentially normal intracellular cation concentration. (Table IV and Fig. 2, *C*). It is of interest to note that only in one instance (H.B.) was the intracellular sodium concentration significantly increased over the control values.

DISCUSSION

The distribution of water and electrolytes in the body fluid compartments of patients in congestive heart failure has received intensive consideration in recent years.[2-4] It is obvious from clinical observations that local differences

*Note that when H.B.$_1$ is compared with H.B.$_2$ intracellular sodium decreased while potassium increased despite the fact that there was no significant change in the serum CO_2.

TABLE III. ANALYSES OF SERUM AND TISSUES IN PATIENTS FOLLOWING TREATMENT

PATIENT AGE	SITE		pH	CO_2 (mM/L.)	H_2O* (GM.)	Cl^{-1}* (MEQ.)	Na^{+1}* (MEQ.)	K^{+1} (MEQ.)	N* (GM.)	FAT (%)
E. $L._2$	Quad.	S†	7.39	26.8	909.5	87.9	123.0	3.14		
42	Fem.	M‡			806.7	22.3	23.0	79.00	27.2	0.7
J. D.‖	Quad.	S	7.34	28.8	905.5	94.7	136.8	5.23		
54	Fem.	M			792.7	17.3	25.3	90.50	24.7	0.4
J. M.‖	Rectus	S	7.41	22.7	929.8	105.3	120.8	5.39		
73	Abd.	M			777.5	17.5	29.0	93.80	27.6	4.6
G. K.‖	Quad.	S	7.36	24.2	910.4	100.6	130.5	3.88		
66	Fem.	M			768.0	13.9	22.5	100.50	31.9	1.8
W. K.‖	Rectus	S	7.45	30.8	899.0	98.0	132.3	5.06		
62	Abd.	M			788.5	19.5	36.6	85.50	30.7	5.3
H. H.‖	Quad.	S	7.44	29.5	911.4	100.1	137.3	4.63		
51	Fem.	M			795.0	25.7	39.7	88.70	28.8	2.8
H. $B._2$	Quad.	S	7.49	33.2	913.9	93.6	136.6	3.25		
59	Fem.	M			813.1	31.1	45.0	81.50	24.5	2.2
S. $S._2$	Quad.	S	7.29	17.8	898.9	94.3	126.6	3.44		
56	Fem.	M			801.8	17.6	25.3	79.5	27.3	
Mean		S	7.39	26.7	907.4	96.8	130.4	4.25		
S.D.§			0.03	4.6	5.9	5.0	7.7	0.88		
Mean		M			792.9	20.6	30.8	87.40	27.8	1.2
S.D.					14.6	5.2	5.6	6.71	2.4	

DIAGNOSES AND REMARKS (TABLE III)

E. $L._2$.—See notes in Table I. This biopsy was obtained after the patient had lost 11.8 kg. of edema fluid. Patient relatively free of edema at time biopsy was taken.

J. D.—Rheumatic heart disease with onset of heart failure nine years previously. Had been edema-free for several years at time biopsy was obtained. Patient on digitalis but had received no diuretics for at least six months prior to biopsy.

J. M.—Arteriosclerotic heart disease with onset of congestive failure six years previously. Patient became edema-free following digitalization, salt restriction, and mercurial diuresis. Had been edema-free one month at time specimen was obtained. Patient was on digitalis and had received no diuretics for four weeks. Specimen obtained under Pentothal anesthesia.

G. K.—Arteriosclerotic heart disease. Onset of congestive failure two years previously. Had been edema-free three months at time of biopsy. Patient on digitalis but had received no diuretics for three months. Specimen obtained under Pentothal anesthesia.

W. K.—Hypertensive cardiovascular disease. Onset of congestive failure eight months previously. Had been edema-free for one week at time biopsy was obtained. Patient on digitalis, but had received no diuretics for one week prior to the time the muscle specimen was obtained under Pentothal anesthesia.

H. H.—Rheumatic heart disease with aortic stenosis. Onset of failure four years previously. Edema-free at time biopsy was obtained. Patient on digitalis, but had received no diuretics for several months prior to biopsy.

H. $B._2$—See notes in Table I. Patient had lost 6 kg. of edema at time this biopsy was obtained. One plus edema persisted.

S. $S._2$—See notes in Table I. Patient had lost 16.4 kg. of edema at time of this biopsy. One plus edema persisted together with a significant degree of ascites. Patient had become acidotic as a result of therapy with cation exchange resins.

*Expressed per kilogram of fresh fat-free tissue.
†Serum.
‡Muscle.
§Standard deviation.
‖Edema-free patients.

in fluid and electrolyte distribution occur since the most marked degrees of edema occur in the dependent portions of the body or in those sites where tissue pressure is at a minimum. Evidence has been presented to suggest a universal decrease in intracellular electrolyte content in heart failure.[2, 4, 5, 6] Despite the fact that these data fit current concepts of congestive heart failure,[2, 5] they are open to criticism.[1, 7, 8]

TABLE IV. DERIVED DATA IN TREATED PATIENTS

PATIENT	$(H_2O)F$ GM./KG. FAT-FREE	$(H_2O)C$ GM./KG. FRESH TISSUE	$\{H_2O\}C$ GM./KG. CELLS	(NA) MEQ./KG.	(K) INTRACELLULAR	(NA + K) WATER	RATIO: (NA)M* (CL)M*	RATIO: (K)M* (N)M†
E. L_2	219.2	587.5	754.6	0.0	135.1	135.1	1.03	2.90
J. D.	157.1	635.6	755.5	4.2	141.0	145.2	1.47	3.67
J. M.	143.9	633.6	741.4	13.6	147.5	161.1	1.65	3.40
G. K.	119.4	648.6	737.5	9.6	154.6	164.2	1.62	3.16
W. K.	169.8	618.7	746.8	20.9	136.7	157.6	2.03	2.79
H. H.	222.2	572.8	738.5	13.8	154.1	167.9	1.55	3.07
H. B_2	288.6	524.3	740.3	8.6	153.5	162.1	1.45	3.00
S. S_2	159.4	642.4	765.7	7.0	122.8	129.8	1.44	3.44
Mean	185.0	607.9	747.7	9.7	143.2	152.9	1.53	3.18
S.D.	51.2	40.4	9.5	6.0	10.6	13.4	0.30	0.30

*Meq./100 Gm. dry fat-free solids.
†Gm./100 Gm. dry fat-free solids.

TABLE V. PHASE MASS DATA FROM PATIENTS BEFORE AND DURING HEART FAILURE

PATIENT		(M) (GM.)	(F) (GM.)	$(H_2O)C$ (GM.)	(S) (GM.)
E. L_1	Initial	1000	165	611	224
	Final	1000	232	606	162
	(M)f	1383	321	838	224
	△	+ 383	+156	+227	
S. S_1	Initial	1000	165	611	224
	Final	1000	282	543	174
	(M)f	1287	363	700	224
	△	+ 287	+198	+ 89	
H. B.	Initial	1000	165	611	224
	Final	1000	289	532	179
	(M)f	1251	362	665	224
	△	+ 251	+197	+ 54	
Mean*	(M)f	1251	308	719	
	S.D. =	± 88	± 62	± 66	
	△	+ 251	+143	+108	

(M) = Extracellular phase plus intracellular phase.
(F) = Extracellular phase.
$(H_2O)c$ = Intracellular water.
(S) = Tissue solids.
Initial = The mass of the phases of one kilogram of muscle from the control patients.[1]
Final = The mass of the phases of one kilogram of muscle from the patient in heart failure.
(M)f = Absolute final weight of the initial phases of muscle after the development of heart failure.
△ = The difference between normal values and the absolute values following heart failure (M)f.
*Mean for all edematous patients.

In the present study attention has been focused on skeletal muscle obtained from edematous areas. The tissues studied by Iseri and associates[5] were obtained at autopsy from the pectoralis major muscle in patients who had died of congestive heart failure. Mokotoff and co-workers[8] and Stock and collaborators[9] analyzed muscle from the gastrocnemius and the quadriceps. The tissues reported by Iseri and colleagues[5] had a water content which was essentially not different from their control values. This was to be expected because of the probable lack of edema in this area. The muscle samples analyzed by Stock and associates[9] from the quadriceps and gastrocnemius were obtained at a time when the patients were essentially free of edema, with one exception, and

as a result the values for water content were not markedly different from the controls. Mokotoff and co-workers[8] reported tissues which were removed in the presence of clinical pitting edema and found a significant increase in total water content.

The patients presented in Table I of the present report all had pitting edema extending to the level of the inguinal ligaments. Assuming that there has been little or no damage to muscle cells as a result of heart failure, the relative changes of the extra- and intracellular phases before and after heart failure have been estimated. The "before" values are the initial values obtained on a series of control patients.[1] The final values are estimated for the phases obtained in one kilogram of muscle removed from the patient in heart failure. Since there is more physiological significance in the absolute changes in the phases than in the relative changes, the disturbances of water distribution in these patients have been calculated in terms of fat-free cell solids per kilogram of original normal skeletal muscle. The calculated phase-volume data for three patients "before" heart failure and "during" heart failure are given in detail in Table V together with the mean values for the other edematous patients studied. These data are illustrated in Fig. 1.

The patients in this group all had large increases in the extracellular fluid volume but also, as demonstrated in Table V and Figs. 1 and 2, *A*, there were large increases in the volume of the intracellular phase. This factor accounts for the decreased concentration of sodium plus potassium in the intracellular phase.

The cations, sodium and potassium, do not account for the total intracellular cationic content, and therefore the total cellular osmotic activity cannot be assessed from these data alone. It is of interest, however, that in three of the specimens reported in Tables I and II the sum of the intracellular concentrations of sodium and potassium is less than that of the interstitial fluid. In five of the cases it is less than the mean value for the combined concentrations of intracellular sodium plus potassium.[1] This deficit in intracellular cation concentration is primarily related to a decrease in intracellular potassium concentration. Such a deficit in cellular potassium content has been suggested by the results of metabolic balance studies in some, but not all, patients recovering from congestive heart failure.[3, 4, 6, 10, 11, 12] Patients W.H., H.B., and L.O. did not demonstrate this deficiency in intracellular cation concentration. The results in these subjects serve to demonstrate that intracellular hypotonicity is not a universal feature of congestive heart failure and that other features, such as alterations in acid-base balance and prior therapy, influence the concentrations of intracellular cations.

Alterations in the fraction of osmotically inactive base, as well as in the total intracellular cation content, have been suggested as factors relating to the cellular hypotonicity of congestive heart failure.[2] The data presented here fail to provide direct information with regard to the amount of intracellular cation which exists in an osmotically inactive form. Data have been presented in the past which demonstrate that movements of water may occur in striated muscle which are independent of the transfers of univalent cations.[13, 14]

In an attempt to explain the disturbances of water and electrolyte distribution in the extra- and intracellular edema of heart failure observed in this study, certain hypothetical changes in (F) and (C) which could be brought about by reasonable changes in the salt and water content of skeletal muscle have been considered. For example, one kilogram of normal muscle as represented by the mean control values and having an assumed concentration of osmotically active substances of 320 milliosmols per kilogram, may be altered in the following ways: (A) addition of water alone, (B) addition of hypotonic salt solution, and (C) addition of isotonic salt solution. The changes in phase-

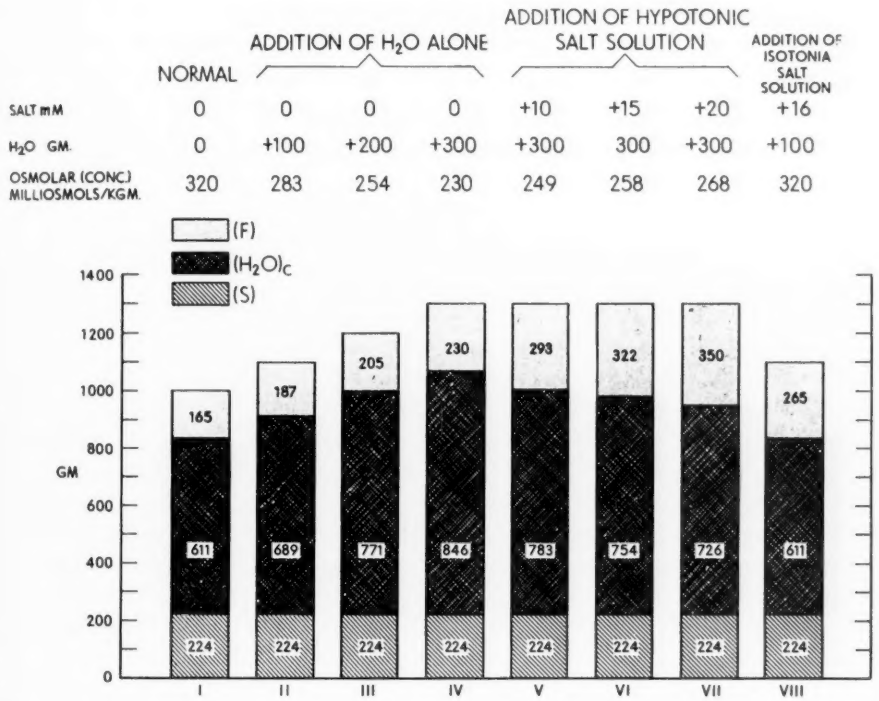

Fig. 3.—Phase-mass data for calculated changes in (F) and (C) of normal muscle which result from theoretical alterations in the water and electrolyte content of tissues. (See text.)

volume distribution as reflected in changes in (F), (C), and $(H_2O)c$ can be calculated under these hypothetical conditions. Such a series of hypothetical conditions are presented in Fig. 3. Column I is representative of one kilogram of normal muscle; Columns II, III, and IV represent the calculated changes that follow the addition of 100, 200, and 300 Gm. of water, but no salt, respectively, to the original kilogram of muscle; Columns V, VI, and VII represent the changes resulting from the addition of hypotonic salt solution in the indicated amounts; and Column VIII represents the changes which occur following the addition of isotonic salt solution. It will be noted that the addition of water to the muscle in increasing amounts results in a proportionate increase in both intra- and extracellular phases (Columns II, III, and IV). The addition of

hypotonic salt solution results in an increase in both intra- and extracellular phases but this increase is characterized by a proportionately greater increase in extracellular phase (F) as compared with the effects of adding a corresponding amount of water (Columns V, VI, and VII). The addition of isotonic salt solution results simply in an expansion of the extracellular phase (F) with no change in intracellular water (H_2O)c. A comparison of these hypothetical cases with the mass-phase data recorded in Table V and in Fig. 1 reveals that the pattern of intra- and extracellular edema of heart failure corresponds most closely to that produced by the addition of hypotonic salt solution to the normal tissues.

Within the limits of the experimental error involved in estimating the volume of the intra- and extracellular phases, the data obtained in edematous patients exhibiting a decreased intracellular cation concentration demonstrate that the primary factor producing this defect was an increase in both intracellular and extracellular water resulting from a hypotonic dilution of the tissue solids.

SUMMARY

Specimens of skeletal muscle obtained from eight patients with cardiac edema and eight patients who had been edematous but who were partially or completely free of edema at the time of the biopsy were studied. The edema of heart failure is characterized by an increase in total tissue water representing an increase in both extra- and intracellular water content. The sodium and chloride content of edematous tissues are increased over control values. This increase is characterized by a proportionately greater increase in tissue chloride than sodium resulting in a decreased Na:Cl ratio in the presence of edema. The decreased intracellular cation concentration observed in edematous muscle is related principally to a decreased intracellular potassium concentration resulting from an increase in intracellular water content and arising from a hypotonic dilution of tissue solids. Treatment of the edema of heart failure leads to a decrease in the water content of intra- and extracellular phases with a concomitant return of intracellular cation concentration to normal levels.

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THE RANGE OF EFFICACY OF SODIUM CHLORIDE SOLUTIONS IN TREATING SEVERE THERMAL INJURY IN THE RAT

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INTRODUCTION

PREVIOUS experience in this laboratory[1, 2] indicated that rats subjected to back burns of 50 to 100 per cent lethality in untreated animals could be successfully treated with intravenous saline therapy. Such results are in accord with those of S. M. Rosenthal's studies on burned mice.[3] However, the applicability of procedures in the rodent to the management of burned human patients immediately raises the question of species differences.

The experments below were designed to determine whether optimum saline therapy in the burned rat would always promote survival. Therefore, in these experiments it was necessary to increase the severity of the thermal injury while at the same time modifying the therapeutic procedure to determine whether an injury could be produced which could not be successfully treated with saline therapy but which would still not be refractory with other types of therapy.

Since it has been possible to realize the objectives set forth above, it is believed that the findings regarding the therapeutic management of severe thermal burns in rodents may have greater significance for the treatment of similarly injured human patients than was formerly believed.

MATERIALS AND METHODS

Unshaved male Wistar rats (weight range, 190 to 210 Gm.), showing no signs of respiratory diseases or gastrointestinal disturbance, were anesthetized with ether and subjected to a back burn[4] in water at 90° C. for thirty-five seconds. Food and water were freely available prior to thermal injury, withheld during the treatment period following injury, and again offered without restriction following a ten-hour postburn interval.

Two series of experiments were conducted, one on rats receiving a 42 ± 2 per cent surface burn, and the other on animals receiving a 50 ± 2 per cent surface burn.

Saline therapy was administered in one of the following three ways: (a) intermittently into the internal saphenous vein (as previously described),[1] (b) continuously through an indwelling polyethylene tube inserted into the posterior facial vein,[5] and (c) by gavage through a number eight French catheter. The intermittent infusions were given at postburn intervals of two, five, and ten hours, or at one-half, two and one-half, five and one-half, and ten and one-half hours. The latter sequence was followed for the gavaged animals. The continuous infusions were begun between two to four minutes post burn, and continued for approximately ten hours.

The saline solution volumes given in these experiments were 10 and 15 per cent of the body weight in one group, 20 per cent of the body weight in a second, and 15 per cent

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of the body weight in four groups. All infusion volumes were calculated from the initial preburn weights of the individual animals within the groups.

The concentrations of the saline solutions used ranged from 0.9 to 3.6 per cent.

Hematocrit samples were obtained from the lateral tail vein as previously described.[1]

RESULTS

The results of these experiments are summarized in Fig. 1. The rats in Groups 1, 2, and 3 each received a 42 ± 2 per cent burn, and the animals in Groups 4, 5, and 6 each received a 50 ± 2 per cent burn. Concomitant burned untreated controls were included in all experimental groups, but are not recorded in the figure. Of the 25 untreated animals receiving burns similar to those in Groups 1, 2, and 3, the majority (96 per cent) were dead at five hours post burn, and all were dead at eight hours post burn. Of a group of 14 untreated rats receiving the 50 ± 2 per cent burn, all were dead at five hours post burn.

Those animals in Group 1 (Fig. 1, Table I) receiving 1.8 per cent NaCl solution showed a consistently lower survival percentage than those infused with 1.4 per cent NaCl solution. At ten days post burn, three of the twelve (25 per cent) 1.4 per cent NaCl infused animals were surviving, whereas none of the 1.8 per cent NaCl animals were alive.

TABLE I. SUMMARY OF PROCEDURE AND SURVIVAL PERCENTAGES FOR RATS IN GROUPS 1 AND 2 OF FIG. 1, BURNED AT 90° C. FOR 35 SECONDS, 42 ± 2% BODY SURFACE

NO. RATS	% NaCl SOLUTION INFUSED	TOTAL % BODY WEIGHT INFUSED	INFUSION INTERVALS POST BURN WITH % BODY WT. INFUSED AT EACH INTERVAL	% SURVIVAL POST BURN				
				1 HR.	8 HR.	12 HR.	3 DAYS	10 DAYS
Group 1								
12	1.8	10	2, 5, 4, 3, 10 hr. 3%	100	67	25	17	0
12	1.4	15	2, 5, 5, 5, 10 hr. 5%	100	83	42	33	25
Group 2								
3	3.6	15	5½, 2.5, 5.5, 10.5 hr. 5, 4, 3, 3%	100	0	0	0	0
3	2.25	15	as above	100	67	67	0	0
12	1.8	15	as above	100	92	92	75	50
12	1.4	15	as above	100	83	83	83	67

Similarly burned animals in Group 2 (Fig. 1, Table I) infused with 1.4 per cent NaCl showed a significantly higher survival percentage (67 per cent) than those in Group 1 (25 per cent). The increased survival percentage followed the procedural changes of beginning the infusions at one-half hour post burn and giving 15 per cent of the body weight in four stages instead of three. It will also be noted that at ten days the 1.8 per cent NaCl infused animals of Group 2 showed a significantly higher (50 per cent) survival percentage than those in Group 1 (0 per cent). Since there is no statistical difference between the survival percentages of the 1.8 per cent NaCl infused rats and the 1.4 per cent NaCl infused rats of Group 2, and since all the animals of Group 2 were burned and managed similarly, the striking increase in efficacy of the 1.8 per cent NaCl therapy reflected in the Group 2 animals can be attributed to the following: a 5 per cent increase in the total infusion volume, beginning of infusions at a shorter interval after burning, and increasing the

number of infusions from three to four. Other rats in Group 2 infused with 2.25 and 3.6 per cent NaCl solutions did not survive beyond twelve hours post burn.

In Group 3 (Fig. 1), twelve rats were gavaged with 4 per cent of the body weight of a 1.4 per cent NaCl solution at one-half hour post burn. They were to receive additional salt solution at two and one-half, five and one-half, and ten

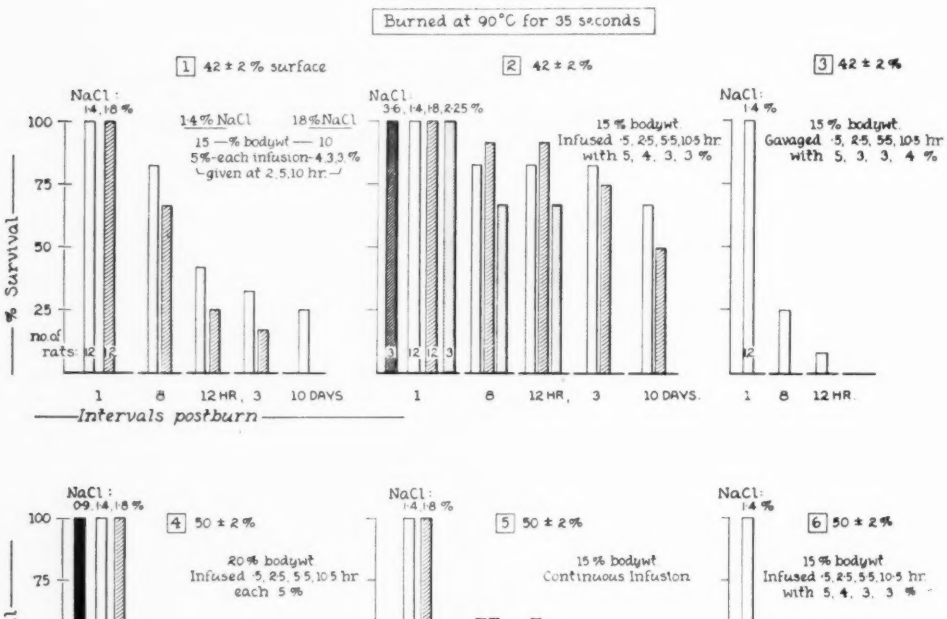
Burned at 90°C for 35 seconds
1 42 ± 2 % surface
2 42 ± 2 %
3 42 ± 2 %
NaCl: 1·4, 1·8 %
1·4 % NaCl
1·8 % NaCl
15 — % body wt — 10
5 % - each infusion - 4, 3, 3 %
given at 2, 5, 10 hr.
NaCl: 3·6, 1·4, 1·8, 2·25 %
15 % body wt
Infused ·5, 2·5, 5·5, 10·5 hr
with 5, 4, 3, 3 %
NaCl: 1·4 %
15 % body wt.
Gavaged ·5, 2·5, 5·5, 10·5 hr
with 5, 3, 3, 4 %
% Survival
100
75
50
25
no. of rats:
1
8
12 HR.
3
10 DAYS.
Intervals post burn
NaCl: 0·9, 1·4, 1·8 %
4 50 ± 2 %
20 % body wt
Infused ·5, 2·5, 5·5, 10·5 hr
each 5 %
NaCl: 1·4, 1·8 %
5 50 ± 2 %
15 % body wt
Continuous Infusion
NaCl: 1·4 %
6 50 ± 2 %
15 % body wt
Infused ·5, 2·5, 5·5, 10·5 hr
with 5, 4, 3, 3 %

Fig. 1.—Survival rates of different groups of burned rats receiving sodium chloride therapy.

and one-half hours post burn in the amounts of 3, 3, and 4 per cent of the body weight, respectively. However, only one animal of this group survived long enough to receive the total volume of 15 per cent of the body weight. Upon postmortem examination all animals in this group showed unabsorbed saline solution in the stomach and small intestine. Several other animals not shown in the figure were gavaged with one-half of the volumes of saline solution recorded above at the same postburn intervals; these animals died between five and ten hours post burn.

Six animals in Group 6 (Fig. 1) receiving 50 per cent burns were infused with 1.4 per cent NaCl solution according to the procedure used for the Group 2 animals. Such a procedure was ineffective; all animals were dead at the end of nine hours post burn. Another series of animals receiving 50 per cent

burns, Group 4, were treated by a similar procedure to that used for Groups 2 and 6. The total infusion volume, however, in Group 4 was increased to 20 per cent of the body weight. Of the three saline solutions infused, 0.9, 1.4, and 1.8 per cent, the 0.9 per cent was the least effective, although none of them promoted postburn survival for as long as twenty-four hours.

In Group 5 (Fig. 1), 50 per cent burned animals received saline therapy by continuous drip begun immediately following the burn. Of the two concentrations of saline infused, 1.4 and 1.8 per cent, the highest survival percentage and longest survival times were obtained following infusions of 1.4 per cent NaCl solution.

DISCUSSION

The results of these experiments show that the survival time of untreated rats is markedly shortened following standardized burns when the injured area is increased from 32 to 42 and 50 per cent of the total body surface. It was also demonstrated that the saline therapy previously shown to be effective in promoting survival of the 32 per cent burned animals[1, 2] was ineffective in promoting comparable numbers of survivors in the more extensive burns. However, when the following procedural modifications in the therapeutic regimen were made, the number of survivors of 42 per cent burns were comparable to those surviving the 32 per cent burns: (a) the infusion volume was increased from 10 per cent of the body weight to 15 per cent, (b) the infusions were begun at thirty minutes post burn instead of two hours post burn, and (c) the infusions were given in four stages instead of three. While the above procedural modifications were found to prolong survival times in rats receiving 42 per cent burns, they were ineffective in promoting survival of rats subjected to 50 per cent burns. However, when the saline infusions were begun immediately and given continuously for ten hours post burn, a 20 per cent survival percentage resulted at ten days. Subsequent investigation[5] showed that 50 per cent burned rats were not refractory to other types of therapy for as many as 75 per cent of similarly burned animals survived when infused with a combination of saline, sodium lactate, whole blood, and plasma.

MacFee and Baldridge[6] found that postoperative shock conditions could be treated successfully in man when large volumes of saline solution were given rapidly. It is interesting that their infusion rates and volumes are comparable to those used for the rats in these experiments.

Recently, Parkins, Perlmutt, and Vars,[7] studying hemorrhagic shock in the dog, have demonstrated that immediate replacement of large volumes of saline was effective in maintaining a good bleeding volume index on repeated hemorrhage, in controlling hematocrit, and in promoting survival. These investigators also demonstrated that saline therapy was of little value in promoting survival in shocked dogs following prolonged hypotension.

A number of other investigators,[8–11] studying other types of shock, have demonstrated the importance of beginning saline therapy early and using frequent intermittent infusions.

Although several investigators[12–15] have proposed the use of oral therapy in the treatment of burns, such a route was found totally ineffective in these

experiments, probably owing to the fact that the burn was of such severity that absorption from the gut was slow. In studies on burned patients, Evans[16] found no special benefit from oral saline.

SUMMARY AND CONCLUSIONS

Rats receiving 42 per cent burns followed by intravenous saline therapy survived for ten days in greatest numbers when infusions were given at one-half, two and one-half, five and one-half, and ten and one-half hours post burn in amounts of 5, 4, 3, and 3 per cent of their initial body weights. The optimum concentration of saline, as indicated by the greatest numbers of survivors, was 1.4 per cent. Oral administration of saline to similarly burned rats, given according to a similar therapeutic regimen, was ineffective.

Rats receiving 50 per cent burns followed by saline infusions according to the therapeutic regimen found to be effective for 42 per cent burned animals died within twenty-four hours post burn.

The survival time of rats receiving 50 per cent burns was increased to as long as ten days for a small percentage of animals by starting infusions immediately post burn and continuing them for ten hours, during which interval 15 per cent of the body weight of a 1.4 per cent NaCl solution was given.

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EXTRAVASCULAR DIFFUSION OF DEXTRAN FROM BLOOD

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AFTER intravenous injection of dextran, the concentration of this material in lymph from the thoracic duct rapidly approaches that of blood; some dextran is excreted in the urine. Macromolecular substances, such as dextran, do not remain entirely within the blood. The high concentration of dextran in lymph suggested that the differential in colloidal osmotic pressure between intravascular and extravascular fluid was minimized rapidly even when the dextran in blood appeared to maintain an increased blood volume. Such observations suggested that dextran in extravascular spaces may influence the availability of fluid for transfer to the circulating blood and that macromolecular substances may affect blood volume by means of other mechanisms in addition to intravascular colloidal osmotic pressure.[1, 2]

The additional experiments that were designed to test this hypothesis and which are reported in this paper indicate that after intravenous injection the concentration of dextran in lymph from the liver is great, whereas the concentration is small in lymph from intestine and muscle. The total amount of extravascular dextran is small and therefore cannot have much influence on blood volume. Analysis of data concerning influx of fluid into the blood after hemorrhage also indicates that extravascular dextran has little influence on mobilization of fluid available to the blood.

METHODS

Rabbits weighing from 3 to 4 kg. were used for the studies on lymph. With the animal under ether anesthesia, the abdomen was opened; the desired lymphatic was exposed and cannulated with polyethylene tubing size P.E. 90. The tubing was tied securely in the lymphatic, and the distal end of the tubing was brought out through a stab wound in the abdominal wall.[3] The thoracic duct was cannulated posterior to the aorta and just below the diaphragm. The lymphatic vessel draining the liver was cannulated a short distance before its entry into the upper portion of the cisterna chyli. The major intestinal lymphatic was cannulated just prior to its entrance into the cisterna chyli. The lymphatic accompanying the right common iliac artery was identified and cannulated in this region. Lymph from this vessel was considered as muscular lymph, although lymph from all structures of the leg is drained by this vessel.

The left femoral artery was cannulated with similar tubing and all specimens of blood were withdrawn subsequently through this tube. Only one lymphatic was intubated in each rabbit, and recovery after repair of the incision was rapid. The rabbit was placed in a confining cage, and lymph was collected for approximately an hour or until the flow of lymph appeared constant. Each rabbit then received 20 ml. per kilogram of body weight of 6 per cent solution of dextran in saline. The total amount was injected in three minutes into the marginal vein of the ear.

For studies of blood volume the rabbits were etherized during cannulation of the femoral artery; the studies were begun approximately one hour after recovery from the anesthetic agent. In most studies, the original blood volume was determined by injection into the marginal otic vein of 2 ml. of a suspension of rabbit's erythrocytes sensitized with radioactive phosphorus.[4] The total circulating volume of erythrocytes was calculated

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from the dilution of these tagged erythrocytes in arterial blood; the volume of plasma was calculated from the arterial cell volume per cent[5] and the total volume of erythrocytes. In other studies, radioactive iodinated albumin[6] was used to determine the volume of plasma by dilution, and the volume of erythrocytes was calculated from the arterial cell volume per cent. Subsequent changes in total blood volume were calculated from determinations of arterial cell volume per cent, with allowance for the amount of blood withdrawn previously. Since the duration of these studies was only four hours, it was considered that changes in the erythrocytic mass were the results of the experimental procedure.

Solution of dextran or isotonic solution of sodium chloride, each in amounts of 20 ml. per kilogram, was injected into the marginal otic vein, and approximately 35 per cent of the total blood volume was removed rapidly by means of the cannula in the femoral artery. In other experiments, approximately 50 per cent of the total blood volume was removed.

Determinations of dextran were made on plasma, lymph and urine by the method of Bloom and Willcox.[7]

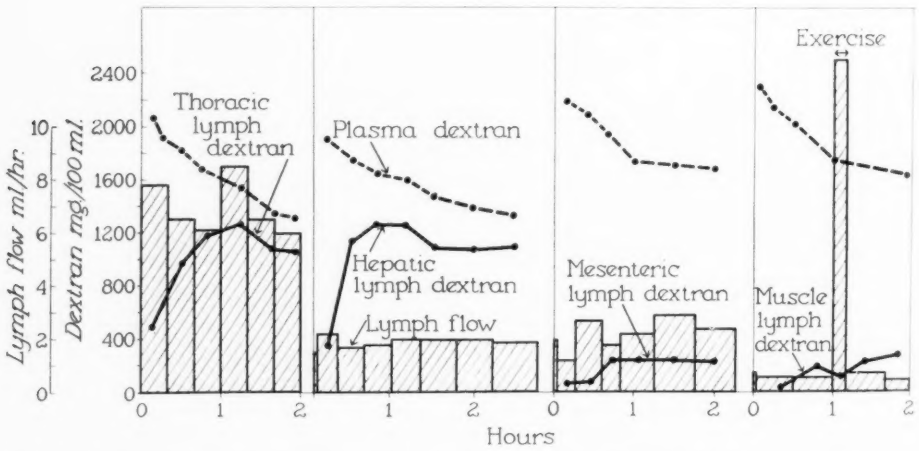

Fig. 1.—Content of dextran in plasma and lymph of rabbits after injection of 20 ml. per kilogram of 6 per cent solution of dextran.

RESULTS

Comparisons of the content of dextran in plasma and lymph are shown in Fig. 1. Each value is the average of the determinations in four satisfactory experiments, the variations in which did not exceed 10 per cent for any single value. Flow of lymph was more variable, but variations in volume of lymph did not appear to affect materially the concentration of dextran in lymph. The average concentration of dextran in lymph from the thoracic duct collected for twenty minutes after administration of dextran was approximately 25 per cent of its average concentration in plasma for the same period; this value for the next twenty minutes was 50 per cent of the concentration in plasma, and it was almost 75 per cent for the next twenty minutes. After one hour, the concentration in lymph reached about 80 to 85 per cent of that in plasma; subsequently, the per cent concentration remained at about the same value as the concentration of dextran in plasma decreased.

The concentration of dextran in hepatic lymph also increased rapidly and remained at a value about 75 per cent of that in blood. The concentration of dextran in mesenteric and in muscular lymph increased within the first hour after injection and subsequently remained at a value from 10 to 15 per cent of that in plasma at comparable periods. Flow of intestinal lymph was relatively small in fasting animals except after oral administration of fluids. In resting animals, flow of lymph from the extremities was small but was increased greatly by movement of the extremities. Passive movement, consisting of flexion of the leg twice each second, produced more than a fiftyfold

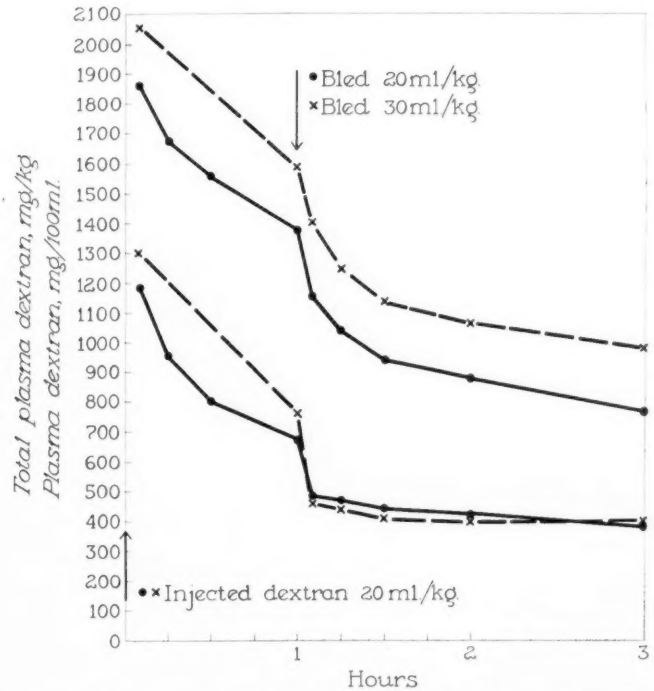

Fig. 2.—Rate of spontaneous disappearance of dextran from plasma of rabbits and effect of bleeding on this disappearance. The two upper curves indicate concentration of dextran per 100 ml. of plasma; the two lower curves indicate the same value multiplied by the volume of plasma per kilogram of body weight.

increase in flow of lymph during the first two or three minutes of exercise. With continuation of exercise beyond this time, flow of lymph diminished to about ten times the resting value; it returned to the resting value almost immediately when rest was resumed. In each of the studies, the concentration of dextran in the lymph during exercise decreased about 30 per cent, while the volume of lymph was greatly increased. Concentration of dextran in lymph obtained during exercise and taken one hour after injection of dextran was about 7 per cent that of plasma.

The blood volume in 20 rabbits prior to injection of dextran was 56.9 (± 1.0) ml. per kilogram, and the plasma volume was 38.4 (± 0.6) ml. per kilogram. Two minutes after completion of the injection, the value for dextran in

plasma was near 2,000 mg. per 100 ml. (Fig. 2) and almost all the injected dextran was present in the plasma. The blood volume was increased by 40 to 45 per cent (Fig. 3) and the plasma volume was increased about 65 per cent. The plasma volume increased 25 per cent more than the volume injected.

One hour after injection, 360 to 420 mg. of dextran per kilogram had been excreted in the urine and 650 to 800 mg. per kilogram remained in the plasma. About 7 or 8 per cent of the dextran could be considered as extravascular. The blood volume was 15 per cent more than the value before injection, and the plasma volume was 28 per cent more. Each gram of dextran remaining in the plasma retained from 12 to 18 ml. of additional fluid.

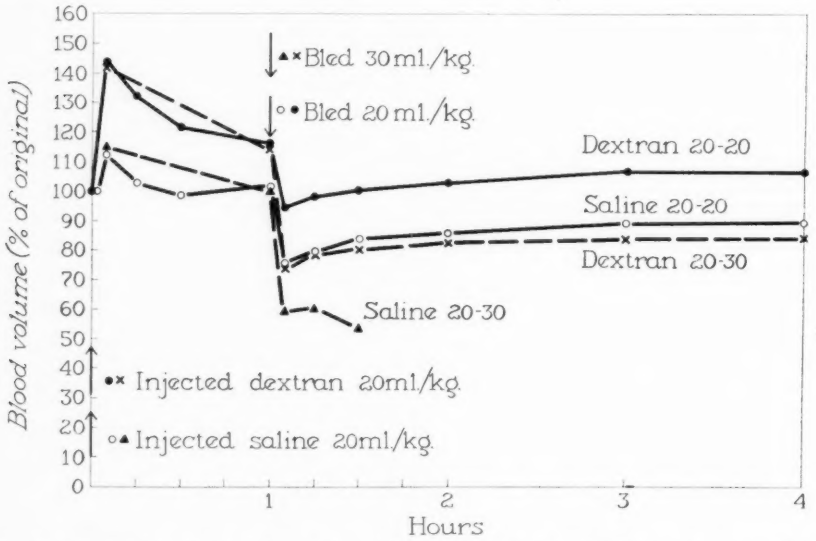

Fig. 3.—Changes in blood volume of rabbits induced by injection of equal amounts of dextran or saline and the effect of subsequent bleeding.

Fourteen rabbits that had received dextran one hour previously were bled rapidly from the femoral artery to the extent of 20 ml. of blood per kilogram. This removed 15 ml. of plasma per kilogram, which amount contained a total of about 200 mg. of dextran; two minutes after completion of the bleeding, a total of about 8 ml. per kilogram of plasma was restored (Fig. 4), with no appreciable restoration of the dextran in plasma. Ten minutes later, an increment of 3 ml. per kilogram of fluid was present in the plasma, and three hours after bleeding an additional 5 ml. per kilogram had been added; during the same period, a gradual diminution of the concentration of dextran in plasma was noted. Changes in total blood volume were similar to changes in plasma volume, but since erythrocytes were not replaced the total blood volume did not return completely to the value present when bleeding was accomplished.

Six rabbits that had received dextran one hour before were bled from the femoral artery to the extent of 30 ml. per kilogram. One died two hours later. The 22.9 ml. per kilogram of plasma thus removed contained 350 mg. of

dextran. Two minutes after bleeding, 6.5 ml. per kilogram of additional fluid had been added to the plasma, with no appreciable increase in content of dextran. Ten minutes later, an increment of 3 ml. per kilogram of fluid was present; gradual influx occurred so that three hours after bleeding a total of 16.5 ml. per kilogram of fluid had been added to the plasma volume. The total influx of fluid under these conditions was almost identical with that found when 20 ml. per kilogram of blood was removed, even though 52 per cent more plasma was removed by means of the more extensive bleeding.

Twelve rabbits received an injection of 20 ml. per kilogram of isotonic solution of sodium chloride. The blood volume before injection was 54.9 (± 0.9) ml. per kilogram, and the plasma volume was 37.6 (± 0.3) ml. per kilogram. Five minutes after the injection, the blood volume was increased by

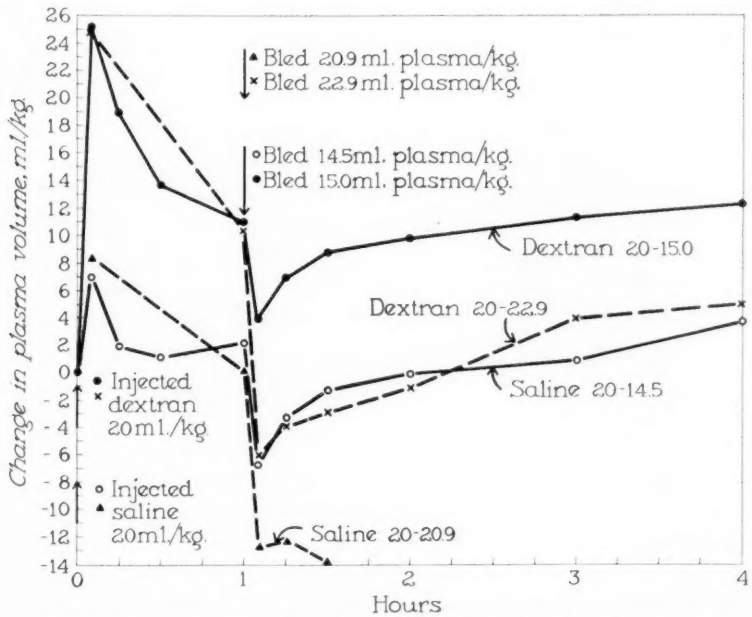

Fig. 4.—Changes in plasma volume of rabbits induced by injection of equal amounts of dextran or saline and the effect of subsequent bleeding.

10 to 20 per cent because of retention in the plasma of 6 to 9 ml. of fluid per kilogram. Ten minutes later, blood and plasma volumes had returned approximately to original values, and only minor changes occurred subsequently.

One hour after the injection of saline, 8 of these rabbits were bled to the extent of 20 ml. per kilogram; this blood contained 14.5 ml. of plasma per kilogram. Two minutes after completion of bleeding, 5.7 ml. per kilogram of plasma had been restored, and ten minutes later 8.7 ml. per kilogram had been replaced. At the end of three hours, 16.0 ml. per kilogram of fluid had been returned to the plasma. The restoration of plasma volume in rabbits treated with saline was not significantly different than that which occurred in similarly bled rabbits that previously had received dextran.

Four rabbits were bled to the extent of 30 ml. per kilogram one hour after they had received saline. Each of these died from thirty to sixty minutes after they were bled. The plasma that was removed amounted to 20.9 ml. per kilogram; two minutes after completion of bleeding, 8.3 ml. per kilogram had been restored. Ten minutes later, 8.9 ml. per kilogram had been restored; thirty minutes after bleeding, plasma volume decreased again as the animals were dying. The influx of fluid into the plasma for the first fifteen minutes was the same as that of similarly bled rabbits that had received dextran.

COMMENT

Diffusion of dextran into mesenteric and muscular lymph is relatively slow and appears to reach an equilibrium with the dextran in plasma in less than one hour. From the values for dextran in lymph flowing rapidly from working muscle it is apparent that the concentration of dextran diffusing from capillaries into extracellular spaces approximates 7 per cent of the amount in plasma. In resting muscle and the mesenteric area, some of the fluid from the extracellular spaces enters the capillaries faster than lymph flows and a small increase in content of dextran in such lymph occurs. In the liver, diffusion of dextran and water into extracellular spaces probably is similar to that from muscular and mesenteric capillaries. The much greater concentration of dextran in hepatic lymph may result from much greater reabsorption of fluid into efferent capillaries because of different gradients of pressure afforded by the presence of hepatic sinusoids. The fact that equilibrium between concentrations of dextran in blood and hepatic lymph is reached rapidly also indicates more rapid turnover of extracellular fluid in the liver than in other tissues.

Most of the dextran that has left the blood within an hour after injection has been excreted in the urine; in these studies, about 100 mg. per kilogram of dextran was in neither blood nor urine. If this amount were present in the extracellular fluid in the concentrations found in lymph from working muscle, the total amount of extravascular fluid containing dextran could be calculated to be from two to three times the volume of circulating plasma. It is obvious that no great store of extravascular dextran is present and there is no indication of an increased extravascular storage of fluid. The influx of fluid into the vascular system after hemorrhage appeared to be similar whether or not dextran was present in the extravascular fluid. In these studies, the blood volume after hemorrhage was greater in those animals that previously received dextran only because the total blood volume was greater before the bleeding occurred. Withdrawal of larger amounts of blood reduced the blood volume to the same level as obtained in animals treated with saline who were bled the lesser amount. Subsequent increases in blood volume in each were then almost identical.

It should also be noted that determination of blood volume at twelve intervals during these studies involved withdrawal of considerable blood in addition to that removed by hemorrhage. In order to wash out the arterial cannula for each specimen of blood and to remove the blood necessary for analysis only slightly less than 1 ml. of blood per kilogram of body weight was

removed at each sampling. Thus, during the three hours, a total of approximately 10 ml. of blood per kilogram was removed. In the calculations, the volume of erythrocytes removed at each period was subtracted from the prior total erythrocytic volume so that the values represent the true volumes present in these animals. In a few animals studied similarly, except that only single preliminary and terminal specimens of blood were taken for analysis, the total changes in blood volume were similar to those that occurred when more specimens were removed. The values for cell volume per cent, however, were greater and the total plasma volume was correspondingly less. Differences occasioned by frequent sampling were evident only after a number of samples had been withdrawn and were not sufficient to alter any of the conclusions.

From the standpoint of maintenance of blood volume after hemorrhage, these studies do not indicate any advantage to be gained by administration of dextran prior to anticipated loss of blood. The slight advantage of the increased blood volume would probably be lost by a correspondingly greater tendency toward bleeding at the time of operation.

SUMMARY

Urinary excretion accounted for most of the dextran that left the blood one hour after injection of this material; only a small amount was retained in extravascular spaces. The concentration of dextran in lymph from the mesenteric area and in lymph from muscle was about one-tenth that of blood. Lymph that flowed rapidly from working muscle contained somewhat less dextran.

Hepatic lymph and lymph from the thoracic duct contained about 75 per cent as much dextran as blood. If the concentration of dextran in the extracellular spaces is assumed to be similar to that of lymph from working muscle, the volume of extracellular fluid may be calculated to be two to three times that of the plasma volume.

Considerable amounts of fluid rapidly entered the blood after acute hemorrhage. Increase in volume of blood after hemorrhage was not influenced materially by the presence of dextran in blood and extravascular fluids before hemorrhage. Changes in volume were identical when similarly bled animals received either saline or dextran before hemorrhage was induced.

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THE MECHANISM OF THE CHANGE IN CEREBROSPINAL FLUID PRESSURE FOLLOWING AN INDUCED CHANGE IN THE VOLUME OF THE FLUID SPACE

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WE HAVE recorded the cerebrospinal fluid pressure continuously for varying periods of time in patients with diverse intracranial disorders. In all patients the recorded pressure seems to be at an equilibrium, for disturbing it by certain mechanisms induces it to return toward its original value. The present communication deals with the mechanism of this displacement and of the return which follows its displacement by the addition of fluid to or the withdrawal of fluid from the cerebrospinal fluid space.

MATERIALS AND METHODS

As wide a variety as possible of patients were studied in order to test the universality of the observations. Most of the patients were on the neurosurgical wards of a general hospital, and were suffering from diverse clinical syndromes (Table I). Some had clinically recovered from their acute conditions; others were in coma. Many had recent or old skull defects. Most were adults. None were given sedation or anesthesia in order to carry out the study. Generally fluid was not withdrawn from or added to the spinal subarachnoid space if the initial pressure was above normal, unless the patient also had a catheter in a lateral ventricle so that fluid could be withdrawn from within the brain, although we have never found it necessary to do so. The pressures in an internal jugular vein, a brachial or femoral artery, and the spinal subarachnoid fluid space were measured continuously, usually with the subject lying on his side (Fig. 1). Increments of spinal fluid were withdrawn, sometimes until the cerebrospinal fluid pressure was below the atrial level by more than 400 mm. Then spinal fluid or sterile normal saline was added in quantities sufficient to elevate the pressure, sometimes to more than 1000 mm. above the atrial level. Following this spinal fluid was again withdrawn until the pressure returned to its original level. Occasionally the order of doing this procedure was reversed. The usual procedure lasted from one to two hours since additional studies were frequently made on the patients.

The pressures in a lateral ventricle, the lumbar subarachnoid space, or internal jugular vein, and a brachial or femoral artery were usually obtained by inserting appropriate saline-filled needles into the proper location according to the techniques of Myerson, Hallo-

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TABLE I. TYPES OF PATIENTS STUDIED

	STUDIED WITHIN ONE WEEK OF INJURY	STUDIED AFTER ONE WEEK OF INJURY
A. *Traumatic*		
1. Cerebral concussion		
a) assoc. with skull fracture	1	3
b) without skull fracture	4	4
2. Cerebral contusion		
a) assoc. with skull fracture	24	10
b) without skull fracture	19	9
3. Hematoma		
a) acute subdural	6	5
b) chronic subdural	0	7
c) epidural	1	1
4. Gunshot wound		
a) of brain	2	0
b) of spinal cord	0	1
5. Miscellaneous	0	7
Total:	57	47
B. *Nontraumatic*		
1. Vascular accidents		17
2. Brain tumors		
a) Primary		13
b) Metastatic		4
c) Pseudo		4
3. Miscellaneous		12
Total:		50
Total number of patients studied: 154		
Total number of procedures: 221		

ran, and Hirsch[5] and Cournand[1] and connecting the needles to sterile saline-filled flexible polyethylene catheters* to strain gauges.† Occasionally, malleable needles were used to record the lumbar pressure with the patient supine.

Clotting was obviated in the needles in the vessels by a continuous leak of heparinized saline (10 units per 500 ml.) at high pressure (400 mm. of saline above the vascular pressure), at a slow rate (1 ml. per minute). This technique was suggested to us by Dr. Bazett. The leak was introduced into the system by a three-way adapter attached to the needle. The extent of the increase in pressure produced by the leak because of the resistance to fluid flow in the needles could be measured by turning off the leak.

The reservoir for the high pressure leak consisted of an oxygen humidifier of 1 quart capacity‡ with the inlet modified by an attachment§ that provided a Luer male tip for the connection of several three-way stopcocks in series. One of the stopcocks was used to control the leak. Another was used to free the system of gas bubbles and to measure the extent of the leak. The latter was done by observing the rate of volume change in a 1 c.c. tuberculin syringe inserted horizontally into the sidearm of the stopcock. The third or fourth stopcocks were used to connect the reservoir to the catheter leading to the vein or to the strain gauge measuring the pressure in the cerebrospinal fluid space. The air in the half-filled reservoir was connected to a leveling bulb by a rubber tube which had a side arm to a rubber handbulb used to modify the pressure in this system. The leveling bulb, half filled with saline or mercury, was leveled at the zero point of a glass tube mounted with a meter rule alongside. This allowed the pressure in the reservoir to be set and measured at any desired point. In addition, the system was used for calibrating the strain gauges. For this reason, it was connected to the gauges measuring the pressures in

*Supplied through the courtesy of the late Dr. H. C. Bazett.

†Models P23-1-135 and P23-6G-240, Statham Laboratories, Inc., 9328 Santa Monica Blvd., Beverly Hills, Calif.

‡Linde 193 Oxygen Humidifier, Ohio Valley Sales Co., Brazee and Enyart St., Cincinnati 9, Ohio.

§Ohio Valley Sales Co.

the cerebrospinal fluid spaces as well as to the blood vessels. All metal-to-metal connections were sealed with sterile petroleum jelly at the start of the procedure in order to avoid the otherwise ubiquitous introduction of ambient air into the system.

The strain gauges were connected to the catheters by three three-way stopcocks with one soldered to one inlet of the gauge, and the other two in series to the other inlet. These stopcocks were used in freeing the gauges from air, in referring the pressure in the catheters to that of the ambient air or of the calibrating reservoir, in adding increments of fluid to the vessels, or withdrawing fluid from or adding fluid to the subarachnoid or ventricular fluid spaces. The pressures in the reservoirs and gauges were all referred to the same "air-zero" which was 10 cm. above the midback when the patient was supine, or at the lumbar spine when the patient was on his side. The fluid level in the reservoirs and the side arms of the stopcocks on the gauges was adjusted to this level. The anteroposterior chest diameter at the angle of Louis was determined by obstetric calipers. When the patient was supine, the "air-zeros" were set at the level of the angle of Louis, less the anteroposterior chest diameter, plus 10 cm.; thus all the pressures were referred to the presumed level of the atrium as zero.

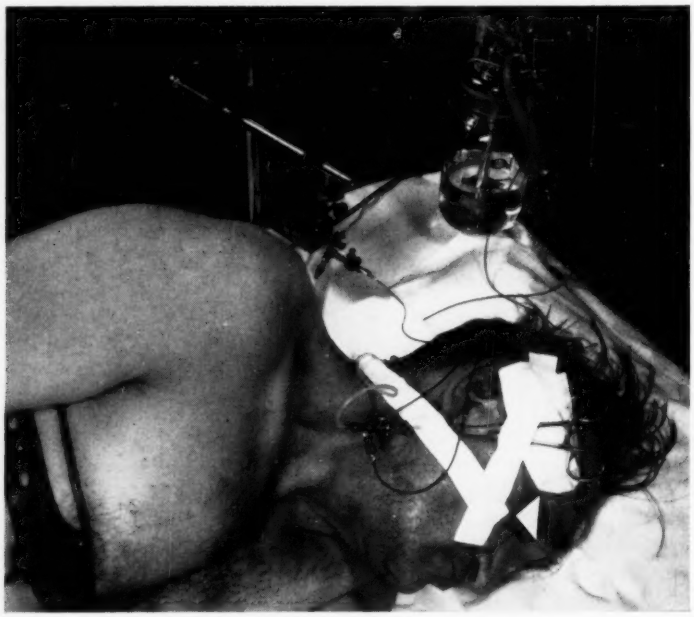

Fig. 1.—The photograph shows the reservoir for the constant leak of heparinized saline, which is also used as a calibrating system. Catheters connect the reservoir and the strain gauge to the stopcocks attached to the needle inserted into the internal jugular vein. A similar system is used for the measurement of the arterial and the spinal pressures.

By the use of appropriate amplifiers* the signals from the pressure transducers were amplified and continuously recorded on two- or six-channel ink-writing oscillographs.† When the amplifiers were properly warmed up, they drifted less than 5 per cent per hour the first hour after they were turned on. The calibration of the gauges was checked at least every hour. A more usual reason than drift for any failure of recalibration to check was the inadvertent introduction of a bubble of air into the catheters or stopcocks leading to the calibrating reservoir. This artefact was prevented by adequate greasing of the metal-to-metal connections and was controlled readily by flushing the catheters with saline. Whenever it was feasible, the lumbar and ventricular pressures were recorded simultane-

*AC-DC Amplifier, Model BL-3108, Brush Development Co., 3405 Perkins Ave., Cleveland 14, Ohio.

†BL-202A and BL-206, Brush.

ously; not only because their correspondence was of physiologic interest, but also because it gave an independent check on the freedom of each recording system from artefact. The use of strain gauges, amplifiers, and ink-writing oscillographs to record the pressures was suggested to us by Dr. John Braunstein.

OBSERVATIONS

When the cerebrospinal fluid pressure was recorded during the withdrawal or the addition of several milliliters of fluid from or to the cerebrospinal fluid space, the rate of the return of the displaced pressure to its original value was maximal immediately or several seconds after the displacement, and the fluid pressure reached its original value within minutes, whether the original pressure was high or normal (Fig. 2).

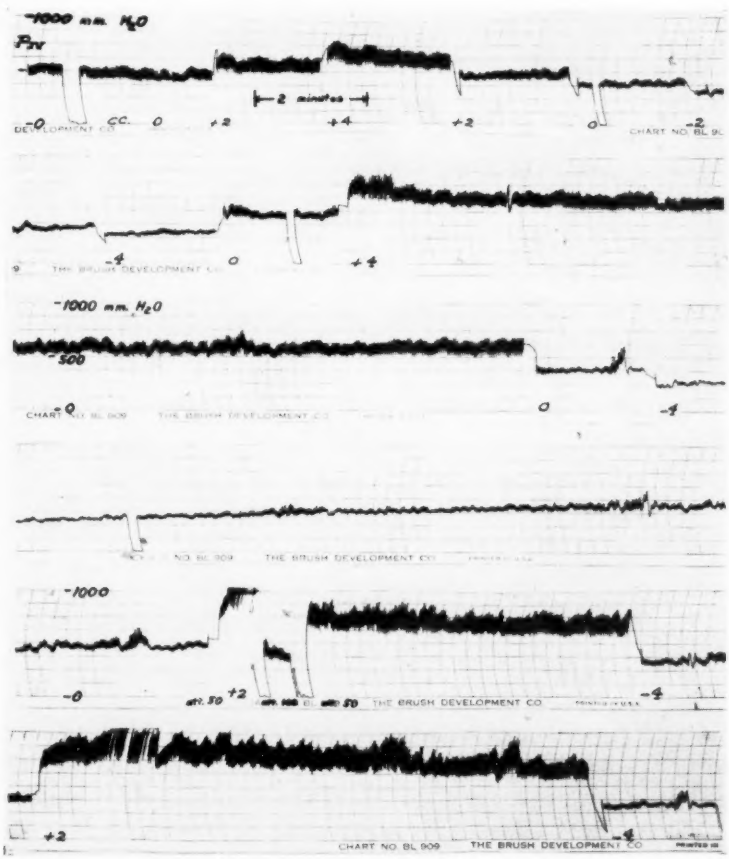

Fig. 2.—The tracing shows the curve of the intraventricular pressure (P_{IV}) in millimeters of water for a period of two hours in a man with multiple metastatic intracranial lesions, a day following an exploratory procedure. The pressures returned toward the original level as fluid was added to or withdrawn from a lateral ventricle.

When large volumes of fluid were added or withdrawn, it was necessary to withdraw fluid from or add fluid to the cerebrospinal fluid space in order to return the pressure to its equilibrium value within a short time. When the intracranial pressure was displaced from its original value by the withdrawal

of fluid from either the lumbar subarachnoid space or the lateral ventricles, the volume of fluid necessary to add in order to return the pressure to its initial value was always less than the volume withdrawn; likewise, when fluid was added to the cerebrospinal fluid space, less needed to be withdrawn to return the pressure to its original equilibrium value (Table II). If the observed differences in the volumes of fluid added to and withdrawn from the cerebrospinal fluid space were due to the net seepage of intracranial fluid out from or into the craniospinal space, it would be expected that the volume differences might have depended upon the maximal induced deviation in pressure, the duration of the deviation in pressure from the original pressure, and possibly on the original pressure and the mean systemic venous pressure. If these variables together modified the volume differences, a first approximation of their interrelationships might be as follows:

$$Y = tP_F^{\Delta} (A + BP_F^O + C\overline{P}_O), \text{ where}$$

Y = expected volume difference in milliliters,
t = duration in minutes of the deviation in the cerebrospinal fluid pressure from the initial pressure,
P_F^{Δ} = maximal induced deviation in fluid pressure,
P_F^O = original fluid pressure,
P_O = fluid pressure at the point of inflection of the pressure-volume curve,[6] which is assumed to be closely related to the mean systemic venous pressure at the craniospinal venous outlets,
A, B, C = constants determined by the data, the regression coefficients.

The experimentally determined values of the regression coefficients calculated from the data of Table II, are as follows:[2]

$$A = .00128 \pm .00022 \quad t = 5.85 \quad P < .001$$
$$B = -.0000037 \pm .0000010 \quad t = 3.57 \quad P = .001$$
$$C = .0000075 \pm .0000024 \quad t = 3.07 \quad P = .01\text{-}.001,$$

which give

$$Y = .00128tP_F^{\Delta} (1 - .0028P_F^O + .0058\ \overline{P}_O)$$

We may conclude that when fluid was added to or withdrawn from the cerebrospinal fluid space, and then withdrawn from or added to the fluid space in amounts sufficient to return the pressure to its original value, there was a difference between the volumes added and withdrawn which was principally determined by the duration and degree of the displacement from the original pressure. There were statistically significant tendencies for the volume difference to be less, the higher the initial pressure, and for the volume difference to be greater, the higher the systemic venous pressure; but these effects did not contribute much to the total variance. A volume difference appeared whenever the equilibrium pressure was disturbed by adding or withdrawing fluid, whether the equilibrium pressure was high or normal, no matter the condition from which the patient studied was suffering, and no matter what the systemic venous pressure was.

PATIENT	"OUTLET" PRESSURE	WITHDRAWAL AND READDITION						ADDITION AND WITHDRAWAL					
		INITIAL PRESSURE	C.C. OUT	MINIMAL PRESSURE	C.C. IN	FINAL PRESSURE	t_1	INITIAL PRESSURE	C.C. IN	MAXIMAL PRESSURE	C.C. OUT	FINAL PRESSURE	t_2
Alex	100	185	50	−160	40	200	29	200	26	1040	8	185	19
Alfz	50	120	25.5	−16	20	124	18	124	22.3	500	7	124	26
Ande (a)	50	150	52.5	−100	40.5	160	26	136	20	970	4	150	14
(b)	55	130	52.5	−115	40.5	140	26	115	20	850	4	130	14
Bass	45							90	30	330	10	90	16
Beau	60	95	10	40	7	90	3						
Bell	112	185	30	17	27.5	200	15						
Bowe	80	300	16	50	14	290	14	220	10	925	2	300	9
Call	84	150	50	−53	35	137	26						
Care	80	210	50	−60	40	200	28						
Chen	45	36	28.5	−91	16	35	11	35	44.5	370	30	51	15
Clea (a)	120	190	25	−300	20	200	14	200	18.5	980	12	215	13
(b)	60	225	29	9	24	238	18	238	15	756	10	238	11
Clem	75	98	70	−160	50	94	45						
Feit	30	132	50	−58	37.5	132	41	132	28.5	1000	2	135	8
Fiel	25	150	6	107	2	150	2	150	16	715	2	150	69
Floy	110	176	30	90	22	164	15	164	20	830	8	170	6
Gard	20	195	20	−24	17.5	190	15	190	5	650	5	120	7
Garr (a)	110	250	63	0	46	260	48						
(b)	50	140	60	−26	40	136	51						
Gask	90	135	10	95	12	132.5	9	132.5	18	350	5	135	19
Gues	65	96	25.9	34	9.9	94	45	94	14	378	8	91	7
Hass	50	420	30	24	10	345	15	450	10	1220	12	440	20
								520	8	1100	8	440	10
Haze	30	112	40	−92	35	112	19						
Henr	20	44	30	−68	22.5	43	22	43	21.5	480	12	43	19
Hunt	110	72	40	−96	20	72	42						
Hupp	30	116	50	−35	40	112	21	112	15	518	8	112	5
Jami	34	300	52.5	−80	46.5	300	37	300	8	620	4	265	15
John	140	170	27.5	−22	25	165	18	165	22.5	520	7.5	180	6
Kize	55	59	20	−2	12.5	56	9	56	35	450	15	94	11
Mont (a)	60	136	55	−56	50	146	21	146	23	590	13	130	23
(b)	50	96	25	20	20	104	11	104	16	430	11	84	16
Moor	40	220	45	−170	30	180	26	180	10.5	920	4	235	10
Murd (a)	50	50	6	20	8	55	6						
(b)	80							45	48	840	16	40	29
McCo (a)	80	290	60	−26	45	250	33						
(b)	110	400	39	84	28.5	398	25						
Neel	115	190	62.5	28	40	200	40	200	30.5	720	10	200	17
Penn	100	165	47.5	20	35	160	31						
Rock	140	136	37.5	70	17.5	134	52						
Stew	100	228	25	74	17.5	224	11	224	7.5	325	2.5	236	4
Weat	35	54	15	13	12.5	53	11	53	15	140	12.5	70	8
Whit	300	500	38	100	38	480	22						
Wood	30	50	50	−160	30	60	11	50	40	1600	30	50	8
Woda	70	240	77.1	−80	57.1	200	56						

The patients were selected from those listed in Table I on the basis of their having sufficient data for the calculation of a pressure-volume curve.[6] The cerebrospinal fluid pressures in millimeters of water are referred to the level of the atrium. The "outlet" pressure is the average pressure at the craniospinal venous outlets and is approximated from the parameter b of the pressure-volume curve. The initial pressure is that immediately before the withdrawal or addition and is considered to be the equilibrium pressure. The time (t) in minutes indicates the duration of the particular displacement.

DISCUSSION

If we assume that the total volume of the intracranial contents was constant at a given pressure, we may infer that some component of intracranial volume compensated for the measured differences in fluid volume before and after the procedure. Foldes and Arrowood[3] have reported that indefinite amounts of fluid can be added to the cerebrospinal fluid space, and Masserman[4] has noted that the withdrawal of spinal fluid can be repeated with the same consecutive patterns of response in the pressures; therefore, the addition or withdrawal of any amount of fluid to the cerebrospinal fluid space leads to net seepage, and some if not all of the volume that compensated for the measured differences in volume added and withdrawn must have been fluid which was induced by the procedures to seep into and out from the craniospinal space.

Since the differences in measured volume at the end of the procedure were due at least in part to the net seepage of fluid, we may inquire as to that portion of the craniospinal space, including its enveloping membranes and case, that compensated for the entire volume change before net seepage occurred, and for the remaining discrepancy in volume while the net seepage was occurring. Of the remaining several components, the vascular bed might be considered sufficiently distensible to allow for a relatively free volume change at a relatively small absolute pressure change.[7] We may assume that changing the volume of the cerebrospinal fluid space immediately induced a reciprocal change in the volume of the craniospinal vascular bed which led to both the change in cerebrospinal fluid pressure and net seepage.

The merits of this assumption are that it is possible and simple: it requires only one variable to respond to a stimulus. If it is correct, no appreciable change occurs necessarily in the total volume within the enveloping membranes and case no matter what the cerebrospinal fluid pressure is, and there are mechanisms by which a change in craniospinal blood volume modifies the cerebrospinal fluid pressure and induces net seepage.

It may be inferred that the original subarachnoid fluid pressure was an equilibrium pressure, in the sense that any fluid exchange into or out from the craniospinal space was balanced. When it was disturbed by the addition of fluid to or the withdrawal of fluid from the cerebrospinal fluid space, net seepage was induced in the direction of compensating for the displacement, and this net seepage continued until the pressure returned to its equilibrium value; therefore, the intracranial fluid volumes are at a dynamic equilibrium, whether the equilibrium pressure is high or normal, no matter the jugular pressure, and no matter the condition from which the patient is suffering. The mechanism for equilibration is essentially the same at high and at normal pressures.

CONCLUSIONS

1. Changing the volume of the cerebrospinal fluid space induces an immediate reciprocal change in the craniospinal blood volume in all men.

2. The change in craniospinal blood volume induces both a change in cerebrospinal fluid pressure and the net seepage of fluid into or out of the craniospinal fluid space in the direction of returning the pressure toward its original value.

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BRONCHOSPIROMETRY

VI. Results of 1,089 Examinations

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INTRODUCTION

PREVIOUS papers of this series have dealt with the historical development and general bibliography on bronchospirometry,[1] the abnormal conditions existing during the test, and the various techniques used for split function studies. Complications of the procedure have been enumerated, and from these a number of contraindications derived. It remains for us to examine the results of the procedure in various abnormal states.

Other efforts in this direction have, for the most part, been devoted to the analysis of observations made in a single clinical syndrome or following a given treatment.[1] It is hoped that the large number of examinations in the present series will make a broader approach possible. An attempt will be made to define the general effects of pulmonary disease on function. The specific effects of pleural, parenchymal, bronchial, and pulmonary vascular disease will be examined separately, and the highly variable results in pulmonary conditions which usually involve several of these factors will be interpreted on the basis of the findings.

The functional changes resulting from medical treatment, pulmonary collapse, or resection, and surgical procedures designed to restore function will also be analyzed in the hope that certain generalizations concerning the effect of treatment on pulmonary function can be made.

An outline of general and specific indications for bronchospirometry will be developed from this material in another report.[2] Individual cases will be

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described who illustrate the commoner respiratory patterns of pulmonary disease. These cases were also selected because they present some of the more frequently encountered indications for bronchospirometry.

MATERIAL FOR STUDY

The material available for analysis comprises 1,089 individual bronchospirometric studies performed at the Physiology Laboratories of the Boston City Hospital, the Sanatorium Division at Mattapan, and the Veterans Administration Hospital at Rutland Heights. Patients were referred from the medical and surgical services of these institutions, and from 34 other hospitals and clinics of four New England states.

TABLE I. PRINCIPAL DIAGNOSES OF PATIENTS SUBJECTED TO BRONCHOSPIROMETRY

	NUMBER	PER CENT
1. Pulmonary disease		
Tuberculosis	883	81.5
Bronchiectasis	26	2.4
Bronchogenic carcinoma	22	2.0
Bronchial asthma	18	1.7
Lung cyst	12	1.1
Pulmonary emphysema complicated by bullae	12	1.1
Atelectasis, ? etiology	9	0.8
Lung abscess	8	0.7
Silicosis	7	0.6
Asymmetrical pulmonary fibrosis due to radiation	6	0.6
Metastatic tumors of the lung	4	0.4
Sarcoidosis	4	0.4
Asbestosis with and without bronchogenic carcinoma	4	0.4
2. Extrapulmonary disease		
Empyema, nontuberculous	24	2.2
Hemothorax and hemopneumothorax	9	0.8
Kyphoscoliosis	4	0.4
Rheumatic heart disease	4	0.4
Tetralogy of Fallot	4	0.4
Diaphragmatic hernia	3	0.3
Constrictive pericarditis	3	0.3
Coarctation of the aorta	2	0.2
Thyroid adenoma, substernal	2	0.2
Other pulmonary vascular congenital anomalies	3	0.3
3. Normal volunteers	19	1.7
Total	1,089	100

The major diagnoses of patients subjected to bronchospirometry are listed in Table I. Pulmonary tuberculosis and its many complications was by far the most frequent indication for split function studies. This predominance was due to a variety of incidental factors and does not truly reflect the actual distribution of indications for the procedure. The data are derived from observations made over a period of four years, and during the first two years the only available facilities for bronchospirometry were in tuberculosis sanatoria. The long period of hospitalization of patients with tuberculosis, and the better organization of sanatoria for long-range study programs facilitated periodic bronchospirometric examinations of these patients. In addition, a number of special projects were undertaken which dealt with the functional changes following collapse therapy. Finally, Sanatorium patients were routinely studied preoperatively, while patients with nontuberculous disease were referred to the laboratories only if special problems existed.

Some of the other groups mentioned in Table I deserve further comment. Studies for bronchial asthma are heavily represented because of a project in which split function studies were carried out before operation and after unilateral and bilateral denervation. Patients

with pulmonary emphysema had bronchospirometry only when manifesting large bullae, and greater impairment of one lung was suspected. A number of the studies of patients with bronchiectasis contributed little to our knowledge of the effect of this disease on pulmonary function because examinations were requested only after pulmonary resection had been performed on one side.

RESULTS

In evaluating the effect of unilateral pulmonary disease our attention is focused on oxygen uptake because it is the final determinant of the productivity of the respiratory effort. The elimination of carbon dioxide is of less interest because it is rarely a problem in unilateral disease. We shall also examine the relationship of oxygen uptake to ventilation and to vital capacity. Finally, the degree of functional impairment determined by bronchospirometry will be compared with the extent of involvement estimated by clinical and other laboratory methods.

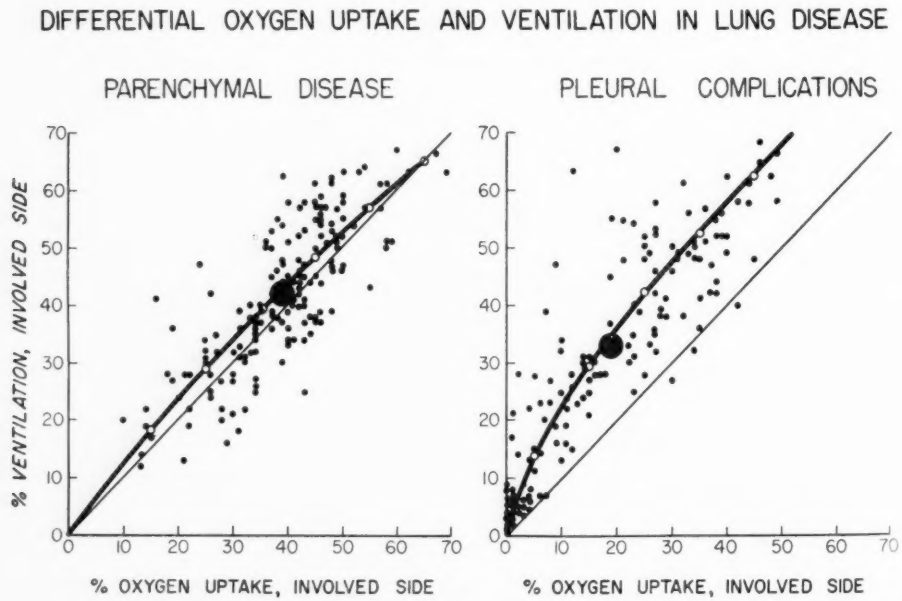

Fig. 1.—The relationship between contribution to total oxygen uptake and to total resting ventilation of 170 lungs with parenchymal disease only and of 127 lungs with functional impairment due to pleural complications. In parenchymal disease oxygen uptake and ventilation are almost equally reduced and average function is only slightly decreased. In the presence of pleural complications oxygen uptake is impaired much more than ventilation and average function is severely decreased.

It is important to distinguish between the effects of parenchymal involvement, pleural complications, bronchial disease, and pulmonary arterial obstruction. Although all four factors are involved to varying degrees in most patients with pulmonary disease, a division has been attempted on the basis of history, physical examination, roentgenographic observation and bronchoscopic findings. To define the effect of a particular type of pulmonary pathology on function, the patients were divided into these four groups. In each a single disorder was

judged to be the primary cause of asymmetrical function on the basis of clinical and laboratory findings. Patients who presented evidence of major involvement by more than one of these categories were excluded.

Parenchymal Disease.—This type of involvement was the principal cause of unilateral functional impairment in 170 patients. Most had pulmonary tuberculosis, although a few patients were included in whom lung tissue was damaged by Friedlander's pneumonia, lung abscess, invasive tumor, or residual fibrosis of unknown etiology. None gave a history of pleurisy or pleural effusion, none had collapse therapy, surgery, or visible bronchial obstruction, none showed "air trapping" on fluoroscopy or evidence of pleural involvement on the roentgenogram.

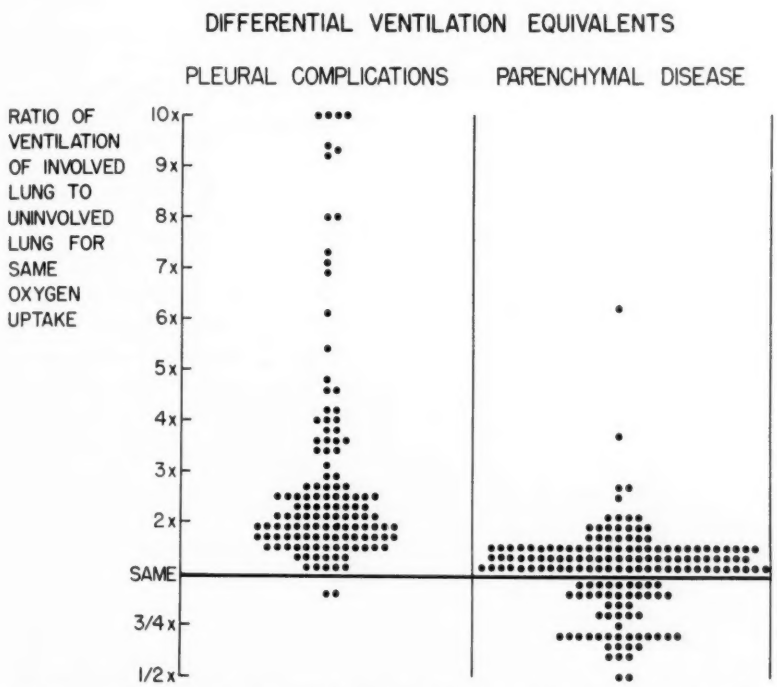

Fig. 2.—Comparison of the ventilation equivalents of the two lungs in unilateral parenchymal disease and in unilateral pleural complications. In lungs with parenchymal involvement the ventilation equivalent is almost the same on the two sides. When pleural complications are present there is marked relative hyperventilation on the involved side. For an identical oxygen uptake the involved lung ventilated up to ten times as much as the uninvolved lung.

The resting oxygen uptake of the damaged lung is compared to its ventilation in Fig. 1. These values are expressed as per cent of total pulmonary function. The fine line at a 45 degree angle to the coordinates describes a theoretical equal reduction of oxygen uptake and ventilation. The heavy line which represents the actual functional contribution in patients with unilateral parenchymal disease deviates only slightly from the predicted equal reduction. The close correlation between impairment of oxygen uptake and ventilation is striking. The actual average loss of function for the entire group is relatively small,

the oxygen uptake being 40 per cent and the ventilation 42 per cent of the total. Many patients show normal functional distribution, and in no instance does the diseased lung contribute less than 10 per cent to the total oxygen uptake or ventilation.

The ventilation equivalent, or the number of liters ventilated for an oxygen uptake of 100 ml., is of particular interest in bronchospirometry. In the absence of hypoxia, it demonstrates the relationship between ventilation and perfusion of each single lung. Fig. 2 demonstrates that this relationship of ventilation to pulmonary arterial blood flow, as expressed by the oxygen uptake, does not differ greatly in the two lungs of patients with unilateral parenchymal disease. In nearly one-third the ventilation equivalent is the same or less on the affected side, and it is rarely more than two times larger on the diseased side than on the well side.

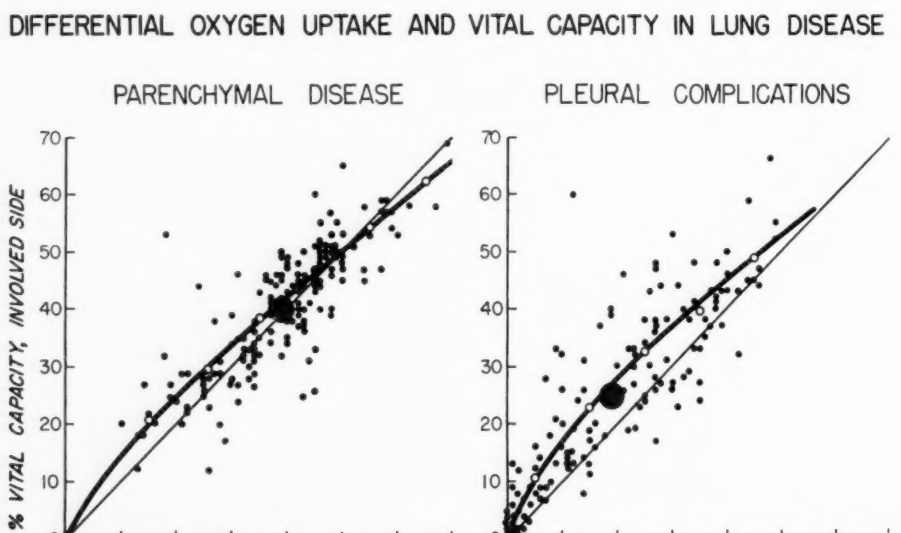

Fig. 3.—The relationship between contribution to total oxygen uptake and total vital capacity in parenchymal and pleural disease.

The relationship between oxygen uptake and vital capacity in the diseased lung is shown in Fig. 3, again expressed as per cent of the total. There is a good correlation and the average participation of all involved lungs in vital capacity and oxygen uptake was 40 per cent.

Pleural Disease and Pleural Complications.—The pleural involvement among the 127 patients in this group was usually the result of therapeutic pneumothorax and its complications, although spontaneous pneumothorax, traumatic hemothorax, pleural effusion, and nontuberculous empyema were included. In many instances there was also some parenchymal involvement such as localized fibrosis. This was thought to have played a minor role in the functional impairment.

The bronchospirometric findings are compared with those of patients with parenchymal disease in Figs. 1, 2, and 3. Fig. 1 demonstrates the striking contrast between pulmonary function in the two groups. In all but three patients with pleural disease the oxygen uptake is more severely impaired than the resting ventilation. In the area of severe insufficiency the heavy line representing the average relationship between ventilation and perfusion bisects the angle between the 45 degree line and the ordinate. This indicates that the lung, severely handicapped by pleural complications, must move about twice as much air than the opposite lung for a given oxygen uptake. The correlation between oxygen uptake and ventilation is less good than in patients with parenchymal disease and predominance of ventilation over oxygen uptake is the rule in the entire group. The average degree of functional impairment is much more severe. The mean oxygen uptake was only 19 per cent of the total and the mean ventilation 33 per cent of the total. Unlike the patients with parenchymal disease 35 per cent of the group with pleural complications had essentially functionless lungs, which contributed less than 10 per cent to the total oxygen uptake.

Eighteen patients had arterial oxygen saturation studies before and during bronchospirometry and a larger number had similar studies before bronchospirometry alone. Only three of these showed unsaturation during the examination. Were this to prevail for the entire group it could be concluded that perfusion and ventilation are about equally decreased in lungs with parenchymal disease but that blood flow is more severely compromised than ventilation in the presence of pleural complications (Fig. 2).

The correlation between oxygen uptake and vital capacity was not as definite as in patients with parenchymal disease. Vital capacity was more impaired than oxygen uptake but less so than resting ventilation. The average oxygen uptake for the group was 20 per cent, the average contribution to total vital capacity was 25 per cent.

In Fig. 4 the 297 patients are subdivided according to type as well as the clinically estimated degree of functional impairment. The predicted normal for each group, indicated by the dotted line, was calculated with consideration for the number of patients with right and left involvement, assuming that the right lung should contribute 55 per cent of the total function and the left 45 per cent. When the degree of parenchymal involvement is estimated from the appearance of the chest roentgenograms, using the classification of the National Tuberculosis Association, a close correlation is observed between the degree of functional impairment and the extent of the disease. Patients with minimal disease showed essentially normal functional distribution, while patients with far-advanced disease showed an average contribution of 30 per cent on the involved side. The roentgenographic appearance in parenchymal disease bears a close relationship to functional impairment, and the actual function is usually somewhat better than anticipated from the appearance of the roentgenogram. These findings are in agreement with those reported by Pinner, Leiner, and Zavod.[3]

The presence of pleural complications completely alters the picture (Fig. 4). In 62 patients who at one time had therapeutic pneumothorax the re-expanded

lung contributed on the average only 30 per cent to the total oxygen uptake. The data obtained following re-expansion do not lead to an entirely fair evaluation of the functional end result following artificial pneumothorax. Only a few patients were studied who were completely well after uncomplicated re-expansion of an effective pneumothorax. The large majority had had extensive disease with only partial true re-expansion and more than half were in the hospital because of incomplete cavity closure or other evidence of activity.

In 41 patients with unexpandable pneumothorax lungs or empyema the involved lung contributed only 10 to 12 per cent to the total oxygen uptake.

Over one-third of 297 patients studied had unilateral pulmonary functional impairment due to therapeutic pneumothorax and its complications. The best performing of the groups with pleural complications showed less oxygen uptake on the involved side than the worst of the three groups with parenchymal disease (Fig. 4). One cannot fail to be impressed by the fact that therapeutic pneumothorax and its complications have been responsible for many of the cases with unilateral functional obliteration seen at present in a sanatorium population. It is fortunate that the era of indiscriminate enthusiasm for this procedure has passed and that fewer patients with damaging pleural complications will be seen in the future. These studies further emphasize the importance of early and vigorous treatment of pleural complications.

Earlier observations of the performance of lungs with pleural involvement[3] were confirmed. The chest roentgenogram offers little help in estimating the functional capacity. Also in contrast to lungs with parenchymal disease, the function of the lung with pleural disease is almost invariably overestimated from the appearance of the chest film.

Some correlation of physiopathologic processes with roentgenographic and bronchospirometric findings is possible for both groups. The roentgenogram in parenchymal disease leads to an unduly pessimistic estimation of function because areas of infiltration, fibrosis, or cavitation are superimposed on normal tissue in the single plane of the chest film. In patients with apparently minimal pleural involvement the function of the lung is overestimated because only a thin rim of pleural thickening is seen along the lateral and superior boundaries of the thoracic cage, whereas exploratory thoracotomy or autopsy shows that this layer of cortex imprisons the lung circumferentially.

The bronchospirometrically determined relationship of oxygen uptake to resting ventilation offers further clarification of the physiopathology in various forms of pulmonary disease. In parenchymal disease the external dimensions of the lung are usually not decreased. In local areas of involvement ventilation and perfusion are equally reduced or eliminated with consequent decrease of function and without accompanying alteration of the ventilation equivalent. If the lung is confined by a cortex its volume is decreased and the cross section of the pulmonary vascular bed is diminished. This leads to increased resistance to pulmonary arterial flow and shunting of the cardiac output to the opposite side. As a result the oxygen uptake of the affected side is severely impaired.

However, during resting ventilation the smallest air passages are not sufficiently reduced to prevent normal or nearly normal ventilation. The inspired air is poorly used and much more ventilation is necessary to produce the same oxygen uptake as a normal lung. Maximal expansion (vital capacity) is sharply limited by the confining layer over the lung and fixation of the thorax and hemidiaphragm. Its tidal volume therefore occupies a much larger share of its vital capacity than occurs normally.

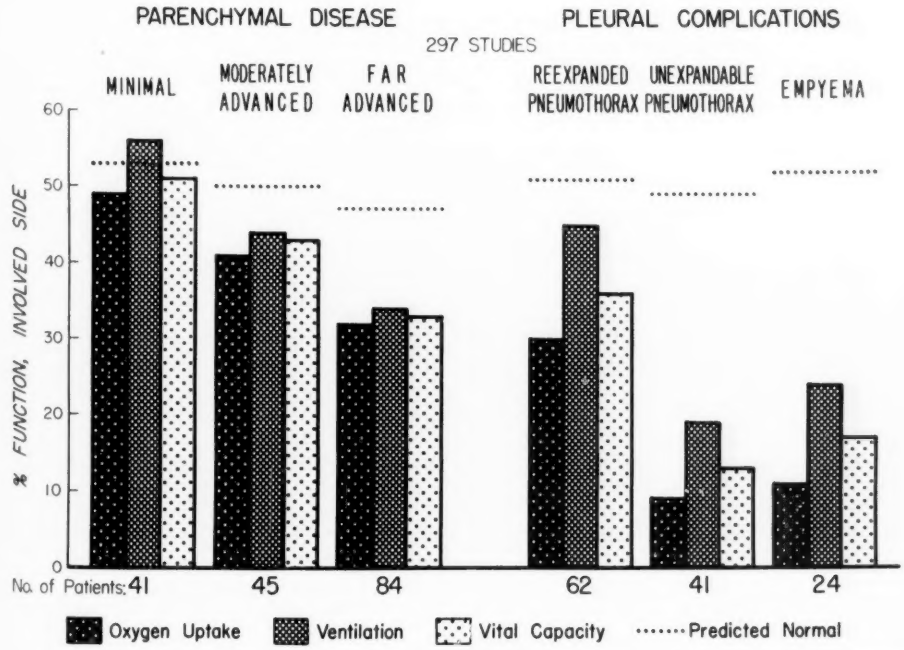

Fig. 4.—Functional participation of lungs with parenchymal involvement and with pleural complications. The predicted normal was calculated by assuming that all right lungs in the group should contribute 55 per cent and all left lungs 45 per cent of the total function. The impairment of function is relatively small in patients with parenchymal disease and the actual degree of impairment is closely related to the extent of disease estimated from the chest roentgenogram. In the presence of pleural complications the functional contribution is greatly diminished and correlation between bronchospirometric data and appearance of roentgenograms is poor.

Bronchial Disease.—Unilateral bronchial involvement causes a greatly variable bronchospirometric picture. If the lumen of a secondary or smaller bronchus is decreased, a localized wheeze may be heard and a small area of "air trapping" may be observed during fluoroscopy. However, bronchial involvement of this type appears to have no effect on the bronchospirometric picture. Even if such involvement progresses to complete occlusion with lobular or segmental atelectasis, the variations do not exceed those of normal functional distribution.

We have studied nineteen patients with varying degrees of involvement of a *major* bronchus due to bronchogenic carcinoma or adenoma in nearly half of the group and due to tuberculosis in the remainder. In nine patients the bronchial

lumen was reduced to a pin point or the obstruction was complete. Their roentgenograms usually showed total atelectasis, and their tracing on that side described a horizontal straight line. Bronchial atresia should be suspected whenever the spirogram for one side lacks even minor volume variations which are due to the changing caliber of the bronchi accompanying changes in intrapleural pressure. These minor excursions persist in destroyed lungs as long as the major bronchi remain patent. In four patients the lumen of the main bronchus was reduced to 2 to 4 mm. These lungs appeared aerated in the roentgenogram, but severe trapping of the air space was noted on fluoroscopy. Over-all function measurements such as maximum breathing capacity, timed vital capacity, and air velocity index were grossly abnormal, although the vital capacity approached the predicted volume. On bronchospirometry, a normal oxygen uptake and ventilation was recorded, and the only evidence of disease was obtained during the vital capacity effort. Characteristically, the uninvolved lung was able to expel its vital capacity volume more rapidly, and a "waiting period" could be observed while the lung with the narrowed bronchus continued to expel air for some time thereafter.[2] If obstruction progresses further, ventilatory excursions and oxygen uptake may be absent at rest, yet on forced inspiration some air may be drawn into the lung which can then be expelled on forced expiration. Often in such cases a smaller volume of air is exhaled under these circumstances than is inhaled, part of the gas being "trapped" as the transverse diameter of the bronchial lumen decreases on forced expiration.[2]

Pulmonary Arterial Obstruction.—The most striking discrepancy between oxygen uptake and ventilation is seen in patients whose lungs ventilate normally but whose pulmonary arterial flow is obstructed on one side. In man complete unilateral pulmonary arterial occlusion has been reported following accidental operative ligation of one main branch of the pulmonary artery, and one branch has been occluded experimentally by a cardiac catheter fitted with a balloon.[1]

We have observed three patients in whom the diagnosis of complete pulmonary arterial obstruction was made by bronchospirometry and confirmed by angiocardiography. The first was a girl with tetralogy of Fallot whose left pulmonary artery was operatively interrupted. The systemic arterial recirculation was apparently insufficient for an appreciable oxygen uptake on the left, and the patient remained cyanotic. A second patient was studied after right upper lobectomy. During operation the right pulmonary artery was injured and repaired. The remaining two lobes contributed 40 per cent to the total ventilation and 38 per cent to the total vital capacity, while there was no oxygen uptake whatever. Occlusion of the right pulmonary artery, apparently due to thrombosis, was later demonstrated by angiocardiography. In the third patient a diagnosis of congenital absence of the right branch of the pulmonary artery was made by angiocardiography. Bronchospirometry with ambient air revealed no oxygen uptake by the right lung, although it contributed 55 per cent to the total ventilation.[4] Unilateral pulmonary arterial obstruction was suspected in two other patients who showed a nearly normal oxygen uptake on the side of thoracoplasty before operation and no oxygen uptake, in spite of adequate ventilation

some months after operation. Arterial obstruction may have resulted from kinking or compression, but this diagnosis could not be confirmed since angiocardiography was not performed.

HYPERVENTILATION RELATIVE TO OXYGEN UPTAKE AND "PARADOXICAL" HYPERVENTILATION

The typical lung involved by parenchymal disease shows a moderate reduction of oxygen uptake, a lesser reduction of ventilation, and a vital capacity which takes an intermediary position. The involved lung consequently shows a slight degree of hyperventilation in relation to its oxygen uptake. The typical lung with pleural involvement exhibits a severe reduction of oxygen uptake, a slight reduction of ventilation and, as in parenchymal disease, a vital capacity reduced between these two extremes (Fig. 4). The result is a more severe degree of hyperventilation relative to oxygen uptake. Early and primary reduction of oxygen uptake is therefore common to all pulmonary disease and was observed in 80 per cent of 1,089 patients studied. Only 48 per cent showed a greater reduction of oxygen uptake than vital capacity.

Discussion of hyperventilation has been confined so far to "relative" hyperventilation, or ventilation which is greater than normal considering the amount of oxygen absorbed. There is another group in whom the ventilation of the affected side was increased not only out of proportion to its oxygen uptake, but out of proportion to its anticipated normal ventilation. In the present series the affected right lung moved more than 55 per cent, or the affected left lung more than 45 per cent of the total ventilation in 147 instances. Often this may have represented deviation from the usual normal functional distribution in patients in whom the disease process had produced little alteration. In some, however, the preponderance of ventilation on the diseased side, in spite of severely diminished oxygen uptake, was so great as to be unmistakable. In others, serial examinations showed the ventilation of the affected lung to be gradually increasing as the oxygen uptake diminished and as the roentgenogram showed progression of disease. Such absolute or "paradoxical" hyperventilation could only be attributed to the disease process itself. We are unable to propose an explanation, either neural or mechanical, to account for this phenomenon.

UNILATERAL PULMONARY INSUFFICIENCY DUE TO SPECIFIC DISEASE

Pulmonary Tuberculosis.—The majority of observations made thus far were derived from patients with predominantly unilateral pulmonary tuberculosis. The one pattern common to all applies not only to tuberculosis but to any pulmonary disease: oxygen uptake is affected first and to the greatest degree, resting ventilation is affected last and to the least extent and vital capacity occupies an intermediary position. In other respects the relationship between pathologic picture and functional behavior is extremely variable in tuberculosis. The functional impairment depends not so much on the "severity" of the disease as on its location. Unilateral pleural or severe bronchial involvement has been shown to cause an early and severe impairment while predominantly

parenchymal disease rarely causes marked functional alterations. In a few patients in whom parenchymal disease progressed to total destruction of one lung the involvement was never confined to one lung.

Chronic Suppurative Disease.—Solitary *lung abscess* produces a functional pattern similar to that seen in cavitary pulmonary tuberculosis. A small or moderately large abscess usually does not involve enough parenchymal tissue to decrease function demonstrably. Multiple or large abscesses, and particularly parenchymal necrosis following certain types of pneumonia, may produce a pattern similar to that observed in far-advanced pulmonary parenchymal tuberculosis.

The damaging effect of *bronchiectasis* is even more difficult to show by bronchospirometry. Unilateral cylindrical bronchiectasis involving one or more basal segments does not appreciably diminish function. Saccular bronchiectasis is rarely seen today. In the few patients we have observed, the condition involved the entire lung and was due to long-standing impaction of a foreign body in the main bronchus, to bronchial destruction by radiation therapy,[2] or to "congenital bronchiectasis."[2] Even after long-standing obstruction the lung may become partially aerated upon removal of a foreign body, but generally remains functionless with respect to oxygen uptake. In the "congenital" type marked hyperventilation relative to oxygen uptake is usually observed.

Pulmonary Neoplasm.—The patient with bronchiogenic carcinoma seen at the laboratory typically has an apparently operable lesion, and the disease is usually complicated by pulmonary emphysema.[2] Small lesions have little effect on pulmonary function unless they cause partial occlusion of a major bronchus with a resulting obstructive pattern. Under these circumstances over-all function studies are more strikingly altered than differential function. The effect of stenosis is slight during quiet breathing, and the effect of obstruction may be masked by the resistance of the catheter during rapid respiratory efforts.

Diffuse Obstructive Disease.—A disproportionately large number of patients with *bronchial asthma* were studied because of our interest in the effect of pulmonary denervation operations. During "quiet" intervals the bronchospirometric pattern appears normal. During an acute attack induced by intravenous injection of methacholine or histamine with the catheter in place the only alterations are an increase in the respiratory rate, a symmetrical decrease in tidal excursions and a slowing of the maximal expiratory effort. Elevation of the base line due to increase in the functional residual capacity usually occurs simultaneously and bilaterally. However, three patients were seen prior to denervation in whom this "trapping" of air occurred on one side only. This was accompanied by a diminution of tidal volume and decrease of oxygen uptake on that side alone.

Patients with *pulmonary emphysema* were studied only when the chest film suggested asymmetrical diminution of pulmonary function due to unilateral bullae.[2] The side with the single or multiple large blebs usually contributed nearly as much to function as the apparently uninvolved side. Postmortem examinations usually showed that these blebs which had been thought to exist

chiefly on one side were in fact diffusely arranged throughout both lungs. The disastrous consequences of spontaneous pneumothorax complicating emphysema are borne out by bronchospirometric findings. Three patients were studied in whom a bleb had ruptured with resulting partial collapse of the lung. In every one of these the involved lung was devoid of function, although multiple adhesions had prevented total collapse.

Pulmonary Cysts.—We have studied the effects of three different types of cysts. Although not wishing to enter the controversy concerning their origin and evolution we feel that, from the physiologic standpoint, the following classification is possible.

In the first group are cysts with apparently widely patent bronchial communication. Over-all ventilatory studies generally show little abnormality beyond slight hyperventilation. The physiologic dead space is increased and split function studies show normal distribution of ventilation and vital capacity and slightly diminished oxygen uptake on the affected side.

In the second and largest group are "tension cysts" with intermittently patent bronchial communication. Over-all function studies characteristically show slight diminution of maximum breathing and vital capacities, a reduced "residual volume," a normal physiologic dead space, and greatly abnormal intrapulmonary mixing. In spite of the large size of these cysts the affected lung usually ventilates nearly as much as the other lung. Since these cysts are essentially closed spaces under positive pressure they act like the air plomb of extrapleural pneumothorax.[5] The motion of the diaphragm and thoracic cage are transmitted by this plomb and exchange adequate volumes of air in the expanded portions of the lung. As in extrapleural pneumothorax the expansion of the cyst appears to take place chiefly at the expense of the residual volume.[5] Oxygen uptake and vital capacity are only slightly diminished.

The third group comprises "cystic disease of the lung," a diffuse process of multiple small cystlike spaces dispersed throughout the lung. In two patients resting and maximal ventilation appeared little disturbed while oxygen uptake was sharply diminished.[2] Apparently blood flow is reduced but nearly normal air exchange continues because the air spaces communicate freely with the bronchial tree.

EFFECTS OF MEDICAL TREATMENT ON DIFFERENTIAL FUNCTION

The number of patients studied after various types of medical treatment is too small and the variety of conditions treated is too large to permit general conclusions. The treatment of pulmonary tuberculosis by artificial pneumothorax is an exception, and the effects of various complications of this form of treatment have been mentioned. The functional result of satisfactory pneumothorax will be discussed in subsequent paragraphs dealing with collapse therapy in general.

Two patients with extensive unilateral atelectasis were studied before and after *electrophrenic respiration.* In both instances the involved lung did not participate in respiration at first, but normal or nearly normal function was

restored after repeated electrical stimulation. However, in one of the two patients fluoroscopy showed some aeration immediately after the first bronchospirometric examination. It was thought that the violent coughing during application of topical anesthesia relieved the obstruction and distal atelectasis.

Two patients with pneumoconioses and two with sarcoidosis were studied before and sometime after treatment with *adrenocorticotropic* hormone. Although the disease appeared to involve predominantly one side and bronchospirometry showed asymmetrical participation, the treatment produced no change in the functional relationship.

Six patients with moderate cylindrical bronchiectasis were studied by bronchospirometry before treatment and after six weeks of intensive *postural drainage* and *chemotherapy*. A contralateral lobectomy had been performed in four of these patients and the treatment was directed toward residual bronchiectasis on the other side. In these as well as in all others in whom lower lobectomy had been performed on one side in the presence of bronchiectasis on both sides, the initial studies showed greatly diminished function on the side of lobectomy. No shift of function occurred following medical treatment. In the two unoperated patients with unilateral bronchiectasis, function was distributed normally before and after treatment.

Four patients were studied before, and two to six months after *breathing exercises* were initiated because of fibrothorax due to artificial pneumothorax complications, traumatic hemothorax and radiation following radical mastectomy. In all four, studies before and after exercises checked within 2 per cent. Recent bronchospirometric studies by Rothstein and Strzelczyk during normal and "unilateral" breathing in trained patients throw some doubt on the effectiveness of breathing exercises.[6] Early and late studies after hemothorax show that the passage of time itself is occasionally attended by significant improvement.[7]

Two patients with inoperable bronchogenic carcinoma were studied before and three months after *radiation* treatment and two patients with the same disease were studied before and three weeks after a course of *nitrogen mustard*. The functional relationship of the two lungs remained unaltered in one patient treated with nitrogen mustard, and considerable improvement of function occurred in the other with clearing of extensive atelectasis. Both patients who received radiation showed less function on the involved side some months after treatment. However, roentgenograms suggested progression of the malignant disease and it was not possible to determine whether loss of function was due to this or due to radiation pneumonitis.

EFFECTS OF SURGICAL TREATMENT ON DIFFERENTIAL FUNCTION

Bronchospirometric and over-all function studies performed before and after thoracic surgical procedures should provide data which would be useful in choosing the ideal operation for a particular type of disease and functional situation. First, the effect of well-standardized procedures, applied to large groups, must be compared. Second, a consideration of the average initial functional participation of each group is necessary. Finally, the individual varia-

tions of the functional results within each group should be defined. When these results are compared with the therapeutic outcome, it should be possible to weigh the advantages of each operation.

When the loss of function after various collapse and resection procedures is to be determined the original functional contribution of the operated lung is an important variable. A radical or extensive procedure performed on a side which is initially nearly functionless causes little over-all loss of function and small alteration in the functional relationship of the two lungs. Conversely,

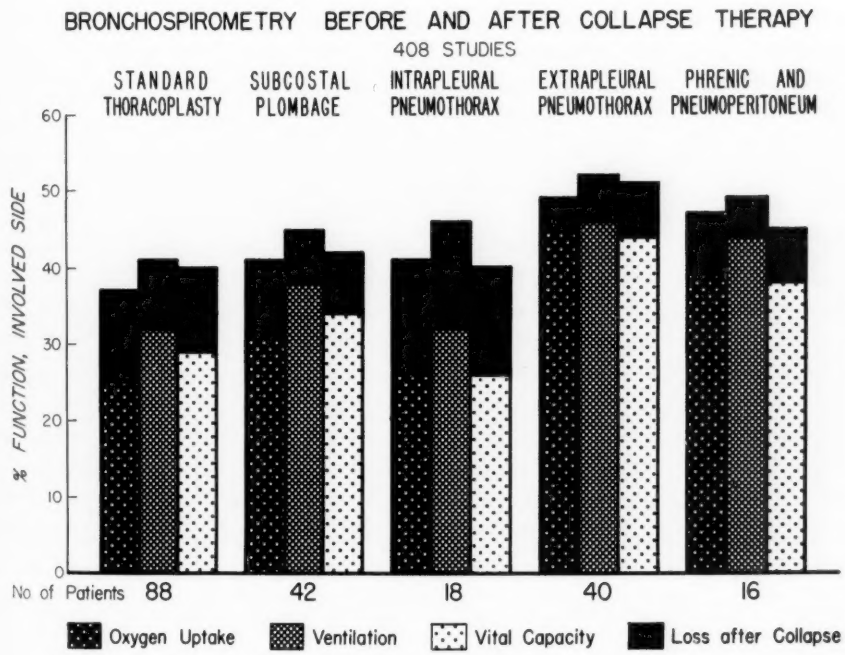

Fig. 5.—Function of the involved lung before and after various collapse measures used in the treatment of pulmonary tuberculosis. Preoperative function of lungs, collapsed by standard thoracoplasty, subcostal plombage, and intrapleural pneumothorax is similar. Plombage causes the least loss and the presence of a pneumothorax space is most deleterious to function. Extrapleural pneumothorax and phrenic paralysis are often performed on the major side and cause little disturbance of function.

even a small collapse or segmental resection performed on a lung which functioned normally causes a considerable drop of over-all function and a marked change in the functional relationship of the two lungs. Finally, when the opposite lung functions poorly, the procedure produces an extensive loss of over-all function, yet the bronchospirometric picture may not change appreciably since the contralateral lung cannot take over a larger share of the total function.

Collapse Therapy.—The results of 408 studies performed upon 204 patients before and after collapse procedures are summarized in Fig. 5. The total height of the blocks shows the mean preoperative contribution to the total oxygen uptake, ventilation, and vital capacity expressed in per cent. The black areas show the mean shift of function to the other side after treatment, and the height of

the remaining coded blocks shows the mean functional contribution of the lung after surgery.

Although no two groups of patients had identical average function before operation, the first three groups who were treated by standard thoracoplasty, subcostal plombage, and intrapleural pneumothorax had lungs which performed nearly equally preoperatively. The decrease of function after subcostal plombage was very slightly less than after standard thoracoplasty. This better end result was quite consistent in all patients and was also confirmed by a lesser diminution of over-all function.[8] Intrapleural pneumothorax caused a shift of function nearly twice as large as that seen after the two types of thoracoplasty. This was true even though the air spaces, maintained during recent years, were never under positive pressure in any phase of respiration and the collapse maintained was never more than 50 per cent. Extrapleural pneumothorax, because it is sparing of function, was often induced on lungs which carried a major share of function. In spite of this the functional decrease was exceedingly small (Fig. 5). Changes in over-all function were also small. The role of the closed air space acting as a "solid" plomb has been mentioned during discussion of tension cysts. A detailed analysis of the various factors responsible for decrease in pulmonary function after these procedures has been presented elsewhere.[5] During the past several years there has been no opportunity to study the effect of unilateral phrenic nerve interruption alone because the operation was always used in conjunction with pneumoperitoneum. Comparison of the data of Fig. 5 with those previously shown for the effect of phrenic paralysis alone[5] suggests that the combined treatment causes more collapse and somewhat larger loss of function. In patients with advanced degrees of pulmonary insufficiency phrenic paralysis with pneumoperitoneum is capable of producing marked respiratory distress.

Pulmonary Resection.—Changes in function after various resection surgery, shown in Fig. 6, are based on 116 studies. Patients chosen for pneumonectomy had poor initial performance on the affected side. The mean contribution of the diseased lung would have been even lower if those with bronchogenic carcinoma had been excluded. One-third of the patients with pulmonary tuberculosis showed less than ten per cent of the total oxygen uptake on the affected side. Lobectomies and segmental resections were performed for well-localized disease. The average initial function was slightly greater than that of the thoracoplasty group. The shift of function to the opposite side was greater after lobectomy than after thoracoplasty. Surprisingly, the loss after resection of one of two segments was often as great as when an entire lobe was removed. This suggests that decrease of pulmonary function following resection is not due to removal of functioning lung tissue as much as to postoperative hemothorax, parietal pleural fixation, and eventual fibrothorax. While this is not always corroborated by the appearance of the late postoperative chest film, there are other observations which do support this interpretation. The decreases of oxygen uptake, ventilation, and vital capacity are almost equal after thoracoplasty. After resection the decrease of oxygen uptake and vital capacity is greater than

the loss of ventilation and this pattern is not prevented by space confining thoracoplasty following resection. Hyperventilation relative to oxygen uptake on the affected side suggests the pattern seen after pleural complications illustrated in Fig. 4. Further evidence of the importance of fibrothorax after resection is given by bronchospirometric data of three patients studied before and some months after exploratory thoracotomy (Fig. 6). While resting ventilation remained nearly unaffected there was a permanent decrease of oxygen uptake and vital capacity. Patients with pleural disease who are subjected to lobectomy and concurrent decortication of the remaining lung may show only negligible loss of function (Fig. 6).

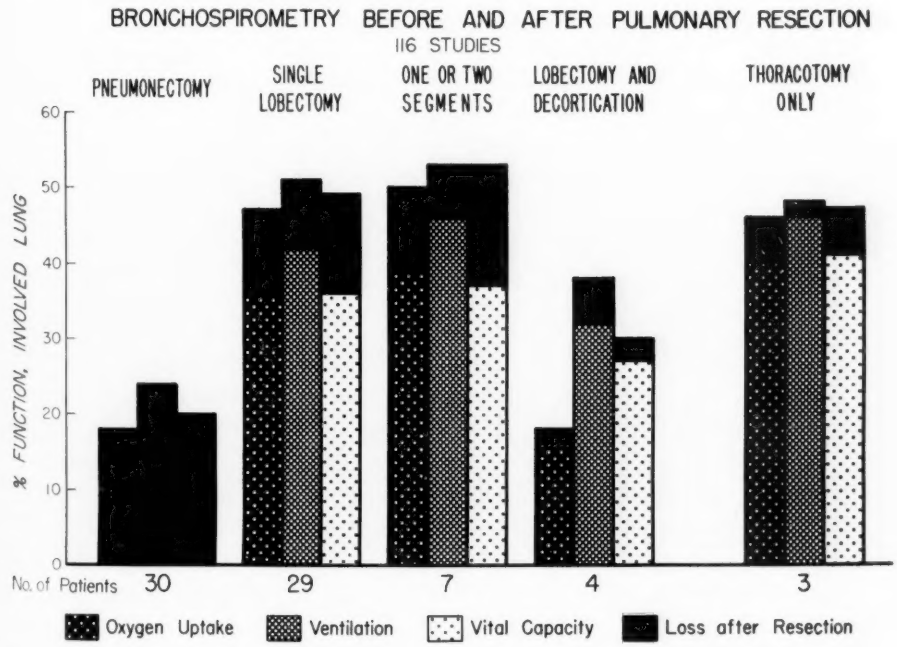

Fig. 6.—Function of the involved lung before and after various types of pulmonary resection.

Fig. 7 was prepared to illustrate individual variation without presentation of lengthy tables. Data of fourteen patients who had right upper lobectomy and twenty-two patients who had right apical six-rib thoracoplasty are presented. The preoperative and postoperative contributions of the affected lung to oxygen uptake and to vital capacity were plotted as per cent of total function. These patients were chosen arbitrarily because together they were the largest single subgroup available, and because it was felt that a right upper lobectomy should cause a loss of functioning lung tissue similar to that due to right six-rib thoracoplasty. The average loss of oxygen uptake and of vital capacity was indeed the same for the two groups. After thoracoplasty there was a moderate functional decrease in every instance with decreases inversely proportional to preoperative impairment. After lobectomy, however, the decreases were much

larger in certain individual patients while in others there was no loss of function or even a small gain. This supports the hypothesis that loss of function after subtotal resection is largely due to attending pleural complications. If these complications are minimized by meticulous hemostasis, frequent postoperative aspiration, and prompt re-expansion of the remaining lobe the end result is excellent. If bleeding occurs in the early postoperative period and the fluid cannot be aspirated, or if the remaining lobe re-expands incompletely, the functional end result may be much worse than after thoracoplasty. The multiplicity of factors which may influence the functional end result of lobectomy is dif-

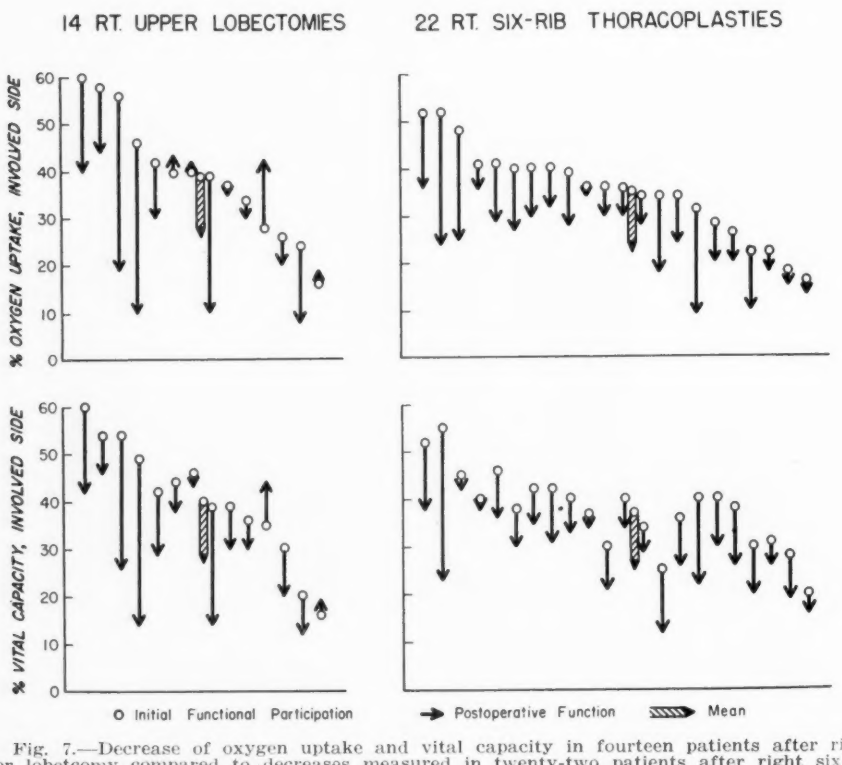

Fig. 7.—Decrease of oxygen uptake and vital capacity in fourteen patients after right upper lobetcomy compared to decreases measured in twenty-two patients after right six-rib thoracoplasty. The average loss is approximately the same for the two groups. After thoracoplasty there is a fairly consistent small reduction. The changes following resection are more variable.

ficult to foresee and the final outcome is highly variable. We are always more confident in predicting the functional result when thoracoplasty is planned.

Reconstructive Surgery.—A number of surgical procedures have been advocated primarily to improve and restore function and may be considered as "reconstructive pulmonary surgery."

Pulmonary decortication is the most frequently employed of these operations. The procedure not only obliterates an infected or potentially infected space but also relieves the severe pulmonary insufficiency due to "pleural com-

plications." We have investigated pulmonary function in fourteen patients before and at intervals up to three years after surgical decortication.[7] Preoperative studies showed a severe restrictive ventilatory insufficiency which was entirely due to a lack of respiratory activity on the affected side. Resting ventilation of the diseased lung was only moderately impaired, as is the rule in pleural complications. Restoration of function after decortication was closely related to the presence or absence of pre-existing parenchymal disease. Patients with little or no parenchymal disease ultimately showed a mean increase of 172 per cent in oxygen uptake, 136 per cent in vital capacity and 53 per cent in resting ventilation by the involved lung. In patients with advanced parenchymal disease the participation of the diseased lung remained the same or decreased further after decortication. The ultimate gain of function was not influenced by the preoperative duration of pulmonary collapse, the presence or absence of infection in the pleural fluid, or the purulence of the fluid. The functional gain bore a close relationship to the amount of re-expansion seen on the chest film. Patients with only visceral decortication showed improvement comparable to that seen in patients with complete visceral and parietal decortication. The results of decortication performed simultaneously with lobectomy, not included in the quoted study, are shown in Fig. 6.

Resection of pulmonary cysts or bullae may also be considered "reconstructive surgery." In two patients with far-advanced bullous emphysema the functional participation of the two lungs remained unaltered after resection of the largest lesions on one side and over-all function was actually decreased. A slight improvement of function on the affected side and a considerable increase of over-all function occurred in three patients in whom "tension cysts" were resected. Unfortunately, the cysts were not large and dramatic changes could not be expected.

Bronchial or tracheal reconstruction is also performed in an attempt to restore pulmonary function. One patient with a bronchogenic carcinoma and one with obstruction due to tuberculosis were studied before and after resection of the involved area and reconstruction of the bronchial tree. In both, over-all studies showed marked improvement of function. The evidence of unilateral obstructive emphysema disappeared: the timed vital capacities and air velocity index returned to normal, the residual volume decreased, and the vital capacity increased, while the total lung capacity remained unchanged. Bronchospirometric tracings were similar before and after operation because the bronchial lumen was large enough to accommodate resting ventilatory exchange. In one patient a marked slowing of the expiratory vital capacity slope was observed on one side before operation which returned to normal later.

Autonomic nerve surgery for the treatment of bronchial asthma may also be regarded as "reconstructive." Four patients with intractable bronchial asthma were studied before and after unilateral pulmonary denervation. Resection of the vagus nerve from the level of the recurrent laryngeal nerve, resection of the anterior and posterior pulmonary plexuses, and interruption of the pulmonary ligament were included. After operation the involved side invariably showed a slight diminution of function which was interpreted as being

due to pleural complications of the extensive exploratory thoracotomy. Two patients were studied before, between, and after bilateral pulmonary denervation. The functional results after these operations were disappointing. Bronchospirometric studies remained essentially unaltered except for small adverse changes due to intrapleural manipulation. Asthma was readily induced after unilateral and after complete bilateral denervation by injection of minute amounts of histamine or methacholine. One of the two patients had a spontaneous attack following bilateral denervation during a bronchospirometric examination. Both patients have since died in status asthmaticus. Much more experimental work and many more studies will be needed to clarify the status of autonomic nerve surgery in the treatment of bronchial asthma and pulmonary emphysema.

SUMMARY

From a series of 1,089 bronchospirometric examinations several groups of patients were selected which, on the basis of clinical and roentgenographic findings, were judged to have unilateral pulmonary involvement of particular anatomic type.

In 170 cases with unilateral parenchymal disease split function studies showed a slight reduction of respiratory participation of the involved lung with oxygen uptake, resting ventilation, and vital capacity almost equally affected. The degree of impairment was less severe than anticipated from the appearance of the chest roentgenogram.

A group of 127 patients with unilateral pleural complications showed a striking diminution of function of the involved lung. Oxygen uptake was most defective, vital capacity was less affected, and ventilation least. In some patients minute ventilation was normal or even increased in spite of severely impaired oxygen uptake. The degree of impairment was more severe than anticipated from the appearance of the chest roentgenogram.

Bronchial disease was responsible for an abnormal distribution of function only when a major bronchus was completely or almost completely occluded.

Obstruction of one branch of the pulmonary artery had little effect on ventilation or vital capacity but oxygen uptake was severely impaired on the affected side.

Bronchospirometric findings in various clinical syndromes and after different types of medical and surgical treatment are discussed in the light of the more general functional patterns.

The loss of function after subtotal pulmonary resection was, on the average, equal to that after thoracoplasty of similar extent. The occurrence of pleural complications following resection made the outcome more variable and hence less predictable than the results after collapse.

Patients were studied before and after surgery designed to improve pulmonary function. Decortication caused marked increase in function if the collapsed lung was not involved by pre-existing parenchymal disease. Some recovery was found following resection of pulmonary cysts. The functional im-

provement after bronchial reconstruction was encouraging. The results of autonomic nerve resection for the treatment of bronchial asthma or pulmonary emphysema were not good.

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BRONCHOSPIROMETRY

VII. Indications

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INTRODUCTION

THE value and limitation of bronchospirometry as a research tool are generally recognized and have been summarized.[1] The place of the procedure in clinical diagnosis is less well defined. Although usually innocuous[2] and capable of precise measurement of the functional contribution of each lung the procedure does require both experience and specialized apparatus. It is emphasized that evaluation of the combined function of both lungs, a necessary preliminary to bronchospirometry, is simpler, conveys information which cannot be obtained by differential studies, and, in many instances, produces all the data necessary for clinical diagnosis and management. It is hoped that the simpler methods for evaluation of ventilatory insufficiency will be available eventually wherever patients are examined. While bronchospirometry has become an accepted procedure in many large medical centers the occasional demands for this procedure do not warrant its introduction to local clinics and small sanatoria. For these institutions referral of patients to near-by chest centers is less expensive and productive of more reliable results.

The cases for this presentation were selected with four objectives: to show the interdependence of over-all and differential function studies; to demonstrate the part played by bronchospirometry in the planning of a therapeutic program; to illustrate certain commonly encountered patterns of pulmonary insufficiency which were evaluated in more general terms in previous publications;[3] and to indicate whether a sufficient number of problems of similar type are encountered elsewhere to warrant introduction of the technique.

MATERIALS AND METHODS

Methods for Over-all Pulmonary Function Studies.—The usual measurements of ventilatory function have been previously described in detail.[4, 5] The maximum breathing capacity (MBC) was collected for thirty seconds with a high velocity respiratory valve and Douglas bag. The volume was measured in a Tissot spirometer and the best of three efforts was reported in liters per minute corrected to BTPS. The vital capacity (VC) and the timed capacities were measured with a modified spirometer used in conjunction with an

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electronic timer.[6] The normal fractions of the total VC exhaled during the first, first two, and first three seconds of the effort were at least 75 per cent, 90 per cent, and 95 per cent of the total, respectively. The per cent of the predicted normal (per cent) VC and MBC were calculated from the regression equations of Baldwin, Cournand, and Richards.[4] The air velocity index expressed the ratio: per cent of predicted MBC/per cent of predicted VC. Values of less than 1.0 were characteristic of obstructive ventilatory insufficiency.[7] Exercise ventilatory requirement was determined during a level walk at the rate of 180 feet per minute as suggested by Warring.[8] The walking dyspnea index was derived from the ratio: 100 × walking ventilation in liters per minute/MBC in liters per minute. These ventilatory studies were obtained in every patient before bronchospirometry. All patients were fluoroscoped to ascertain the extent of motion of the diaphragm and thoracic cage, the presence of "trapping" and of mediastinal motion.

The residual volume, total lung capacity, intrapulmonary mixing index, and spirogram were obtained in duplicate for all patients seen at the Thorndike Laboratory. At the Sanatorium Division these measurements were made only when there was other evidence of obstructive ventilatory insufficiency. Studies were repeated after bronchodilator aerosol if bronchial asthma was suspected. These and other more complex studies will be mentioned only if their outcome was important for clinical management.

RESULTS

The indications for 1,089 bronchospirometric examinations are listed in Table I. Ideally, every examination should aid in the management of the individual patient's clinical problem and at the same time provide cumulative material for the study of a certain disease or particular treatment. This double objective was not always achieved. Many patients were examined because of our interest in a special problem. Such studies were primarily "investigational." Other patients were seen by request of their physicians because future treatment depended largely on the outcome of pulmonary function studies. These examinations were classified under "medical" or "surgical" indications. Many of the results later added to our knowledge of disease and the effect of treatment in general.

I. Investigational Indications.—The principal purpose of 777 studies was the investigation of the effect of disease or treatment on the respiratory apparatus. Although the outcome of such an examination usually did not influence the treatment of the particular patient, the accumulated data were analyzed and reported and the management and treatment of subsequent patients were influenced by the outcome of these studies.[3]

Since 71 per cent of all studies were performed to gather general information it has been suggested that bronchospirometry is primarily a research tool. However, 311 patients were referred from a small section of the country during a short period for the sole purpose of clinical evaluation. We believe that the procedure will eventually find its widest application in this field. Its value for research is secondary and its use in this connection will depend on the inclinations of the personnel responsible for the examination.

II. Medical Indications.—Bronchospirometry is seldom requested unless surgery is contemplated. Split function study has occasionally been part of a battery of examinations performed to investigate dyspnea or cyanosis of unknown etiology in diffuse pulmonary disease which appeared to predominate on one side (Table I). The fourth to sixth medical indications of Table I refer

TABLE I

PRINCIPAL INDICATIONS FOR BRONCHOSPIROMETRY

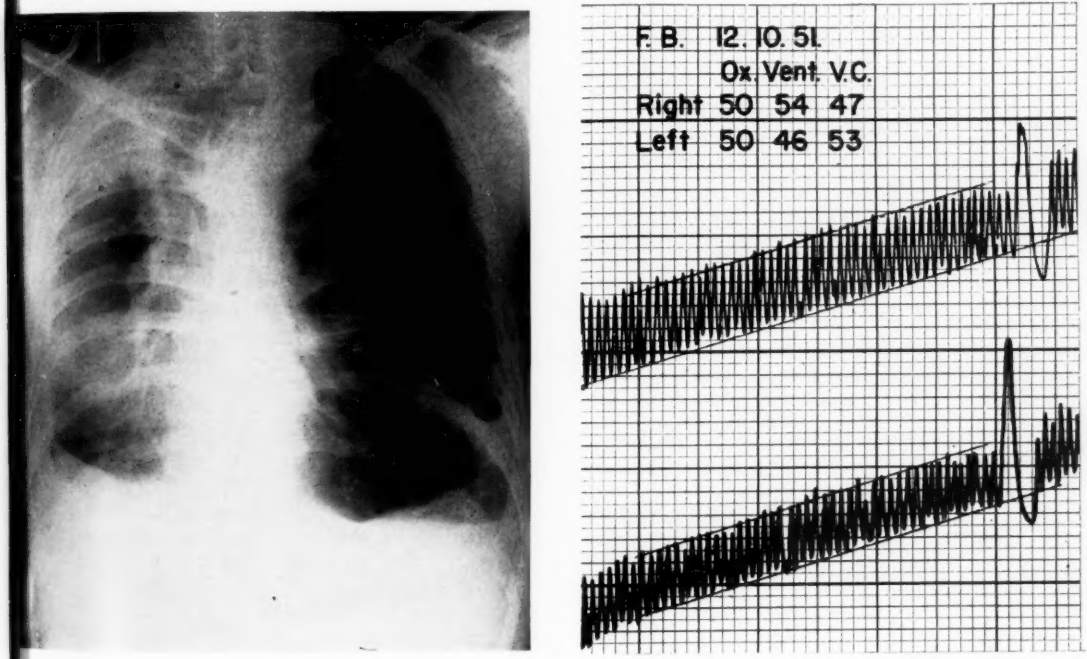
	Number	
I. *Investigational*		
Routine preoperative studies	264	
Routine postoperative studies	284	
Differential function during surgery	150	
Routine studies before medical collapse	32	
Routine studies after medical collapse	29	
Special studies on normal volunteers	19	
Total, investigational	778	71.4%
II. *Medical*		
Investigation of predominantly unilateral disease	12	
For investigation of dyspnea, ? etiology	7	
For investigation of cyanosis, ? etiology	1	
Before medical collapse in dyspneic patients	6	
Before medical collapse in patients with previous contralateral disease or collapse	4	
Before bilateral medical collapse	2	
Investigation of congenital cardiovascular anomalies	9	
Examination before and after medical treatment for predominantly unilateral disease:		
a. Atelectasis after electrophrenic respiration	2	
b. Pneumonoconioses after ACTH	4	
c. Bronchiectasis after postural drainage and chemotherapy	6	
d. Fibrothorax after breathing exercises	4	
e. Inoperable carcinoma after radiation or nitrogen mustard	6	
Total, medical	63	5.8%
III. *Surgical*		
Before surgery in patients with previous medical collapse or surgery on the opposite side	65	
Before surgery in patients complaining of dyspnea	28	
Before surgery designed to improve asthma, emphysema or fibrosis	22	
Before surgery in patients with bilateral disease	13	
Before surgery in patients with previous contralateral disease	11	
Before planned bilateral surgery	11	
To determine the extent of surgery	24	
To determine asymmetry of function in apparently unilateral disease	8	
To evaluate the success of "reconstructive" chest surgery:		
a. After pulmonary decortication	36	
b. After resection of cysts or bullae	9	
c. After bronchial reconstruction	4	
d. After autonomic nerve surgery in the treatment of emphysema and asthma	16	
e. After diaphragmatic herniorrhaphy	1	
Total, surgical	248	22.8%

chiefly to therapeutic pneumothorax. A number of examinations were performed before and after medical treatment for predominantly unilateral disease. The results of treatment by electrophrenic respiration, adrenocorticotropic hormone, postural drainage, chemotherapy, breathing exercises, radiation therapy, and nitrogen mustard have been quoted previously.[3] In patients with congenital cardiovascular anomalies, particularly of the pulmonary arterial supply and in intrapulmonary arteriovenous aneurysms, bronchospirometry may on rare occasions be of supplementary value.

III. Surgical Indications.—By far the largest number of patients referred for evaluation were seen prior to surgical collapse or resection or before and after operations designed to improve pulmonary function. An attempt has been made to classify the "surgical" indications into a relatively small number of groups (Table I). Undoubtedly, problems have been encountered by others

which do not readily fit any of the categories mentioned. Fifteen of the 248 patients seen before operation were selected for individual presentation. They were representative of the more frequently encountered indications. The place of bronchospirometry in clinical management is better defined by these case histories than by lengthy discussion of the general headings.

Bronchospirometry in Patients With Previous Collapse or Resection on the Opposite Side.—The greatest number of patients who had split function studies before surgery were referred because of previous contralateral medical or surgical collapse or some intrapleural surgical procedure. Most of them were not



A. *B.*

Fig. 1.—Roentgenogram and bronchospirogram of F. B., a 44-year-old man with active pulmonary tuberculosis, a re-expanded therapeutic pneumothorax on the right and an unexpandable lung with an apical cavity on the left. Surgery was contemplated on the left side. The MBC was 67 liters per minute (56 per cent) and the VC was 1,900 ml. (48 per cent). The lung to be operated upon contributed one-half of the total function.

short of breath, but their roentgenograms suggested that the side to be operated upon was the major contributor to pulmonary function. Poor re-expansion following treatment by artificial pneumothorax was the most frequently encountered contralateral situation:

F. B., a 44-year-old man, was referred from the Rhode Island State Sanatorium. There was a seventeen-year history of pulmonary tuberculosis. Therapeutic pneumothorax had been induced on the right sixteen years before and was maintained for seven years. Therapeutic pneumothorax on the left side had been continued for nine years, and the lung failed to re-

expand when refills were discontinued. A cavity persisted at the apex of the left lung (Fig. 1).

Pulmonary function studies revealed an MBC of 67 L. per minute (56 per cent) and a VC of 1,900 ml. (48 per cent) with normal timing. The walking ventilation was normal and the walking dyspnea index was 28 per cent. These results suggested a marked ventilatory insufficiency of the restrictive type.

In spite of obliteration of the space on the right and a persisting pneumothorax on the left, bronchospirometry showed an approximately symmetrical functional distribution (Fig. 1). There was false re-expansion on the right, and pleural symphysis had taken place at the expense of mediastinal shift.

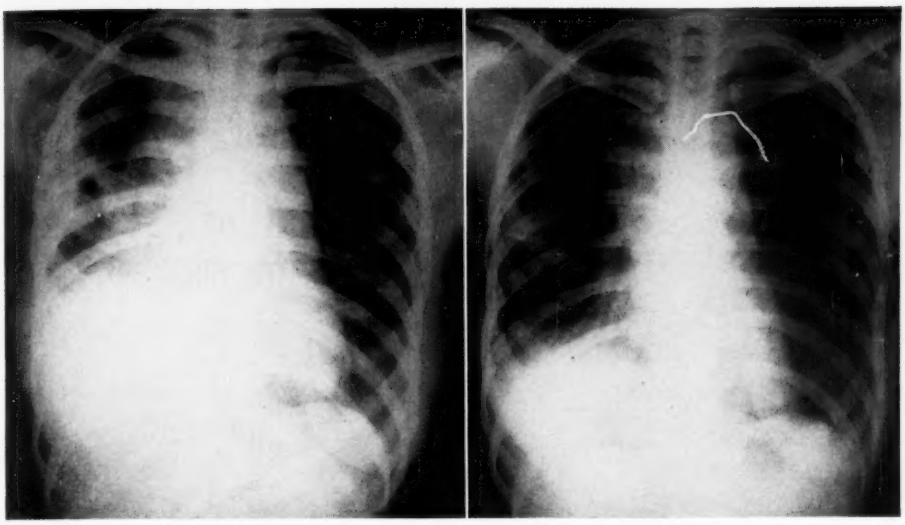
In view of the marked pulmonary insufficiency, it was felt that decortication prior to, or simultaneously with, collapse or resection was indicated.

If pulmonary insufficiency is clinically evident, hesitancy in operating on the side which appears to contribute more than half of the respiratory effort is justifiable. Poor function need not be due to previous contralateral surgery. Pleural effusion due to cardiac failure with incomplete pulmonary re-expansion on the right side has suggested split function studies before a left transthoracic approach for the repair of various cardiac disorders. Occasionally, a bilateral approach becomes necessary. Pulmonary function studies including bronchospirometry may be useful in determining the proper interval between operations. In two patients with mitral stenosis an interauricular septal defect was surgically created through a right transthoracic approach to relieve the pulmonary congestion. When this induced Lutembacher's syndrome failed to produce the desired relief, split function suggested the proper timing for mitral valvuloplasty through the left thorax. A similar situation was encountered in a patient who eventually required a bilateral exploration for removal of a mediastinal thyroid adenoma:

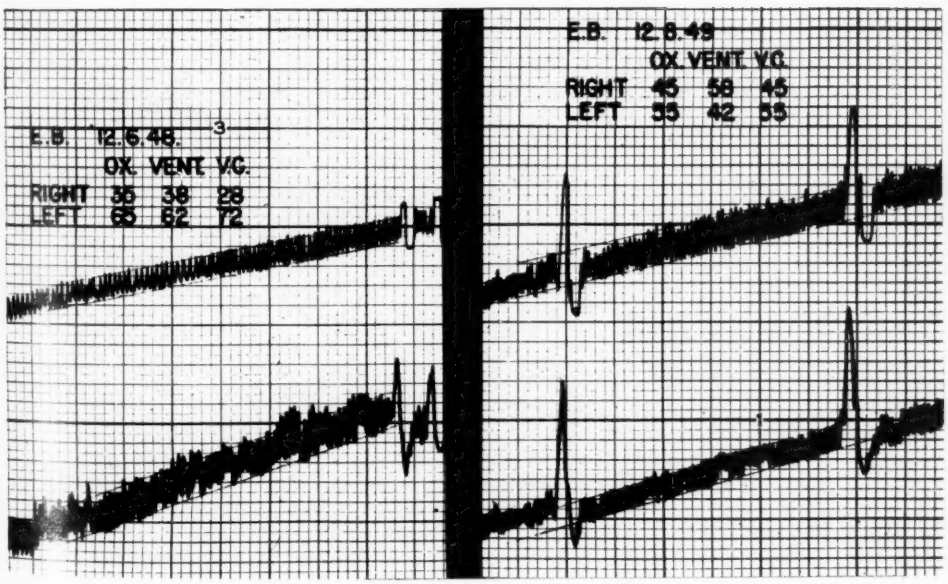
E. B., a 25-year-old woman, was referred from the Massachusetts Memorial Hospitals. She had a large mediastinal thyroid adenoma. A portion of the tumor was removed by a transpleural operation on the right. A similar operation was planned on the left to resect the remaining tumor. Function studies were obtained because of exertional dyspnea and incomplete re-expansion of the right lung (Fig. 2).

Routine studies showed a severe restrictive ventilatory insufficiency, with an MBC of 51 L. per minute (57 per cent) and a VC of 1,260 ml. (39 per cent). Bronchospirometry localized the defect to the right lung (Fig. 2). Since the patient would have depended on this side during a left thoracotomy, and for some time afterward, the operation was not undertaken. The patient was seen again one year later, at which time her MBC had returned to 96 L. per minute (104 per cent), and her VC had improved to 1,750 ml. (55 per cent). Bronchospirometry showed functional recovery of the right lung, although oxygen uptake and vital capacity remained below normal (Fig. 2). This gradual improvement over a period of months or years is commonly observed following thoracotomy.[3] The characteristic discrepancy between oxygen uptake and ventilation, commonly seen in pleural complications,[3] was also observed. Recovery of function was partly due to disappearance of postoperative pain and increased strength. Bronchospirometry indicated that clearing of the pleural effusion and partial absorption of intrapleural hematoma also contributed to the improvement. After the second study it was felt that left exploratory thoracotomy would be tolerated without difficulty.

The problem may become very complex when surgery is planned for a patient who has had previous operations on both sides:



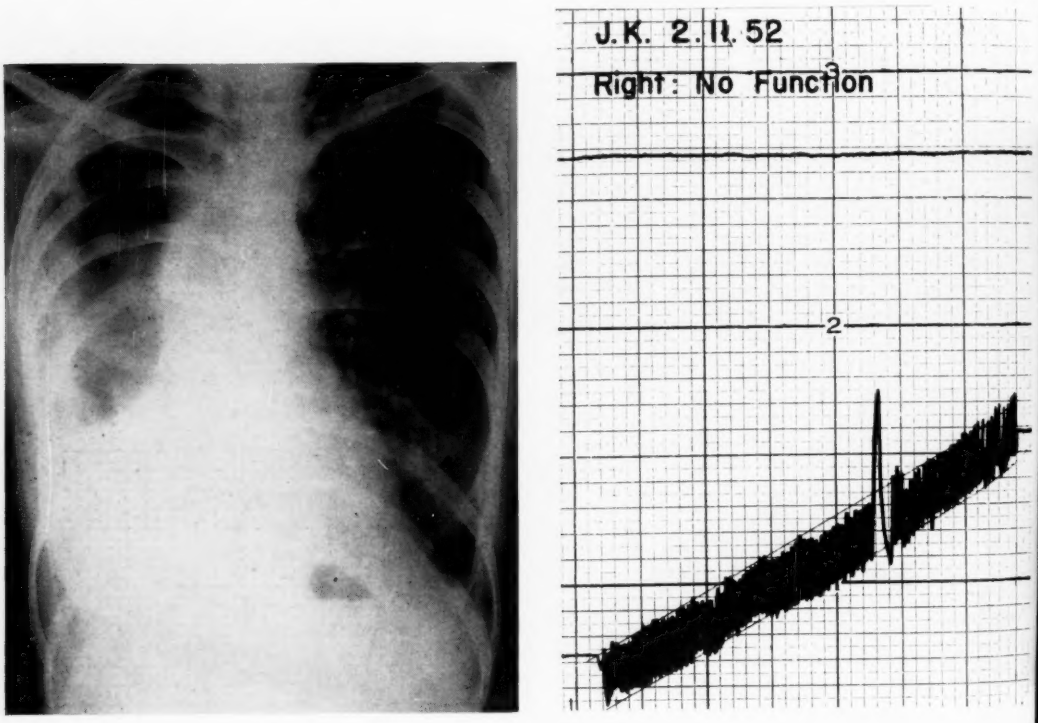
A.



B.

Fig. 2.—Roentgenograms and bronchospirograms of E. B., a 25-year-old woman with a large substernal thyroid adenoma. The first film and tracing were obtained three weeks after a right thoracotomy. Because of poor over-all function and the major contribution by the left lung, a left thoracotomy for removal of the remaining tumor was deferred. The second film and tracing were obtained one year later. At that time over-all function was greatly increased and the right lung had resumed a nearly normal share of the total function. Left thoracotomy was deemed safe.

J. K., a 24-year-old woman, was referred from the Worcester City Hospital about one year after bilateral surgery for bronchiectasis. An uneventful left lower lobectomy had been followed by a right lower lobectomy which was complicated by bronchopleural fistula, empyema, and partial collapse of the right, upper, and middle lobes (Fig. 3). Following drainage of the empyema the patient complained of severe exertional dyspnea. Closure of the bronchial defect and decortication of the right upper and middle lobes was planned. Because it was feared that this procedure might fail, it was important to determine what risk of further functional impairment was involved.



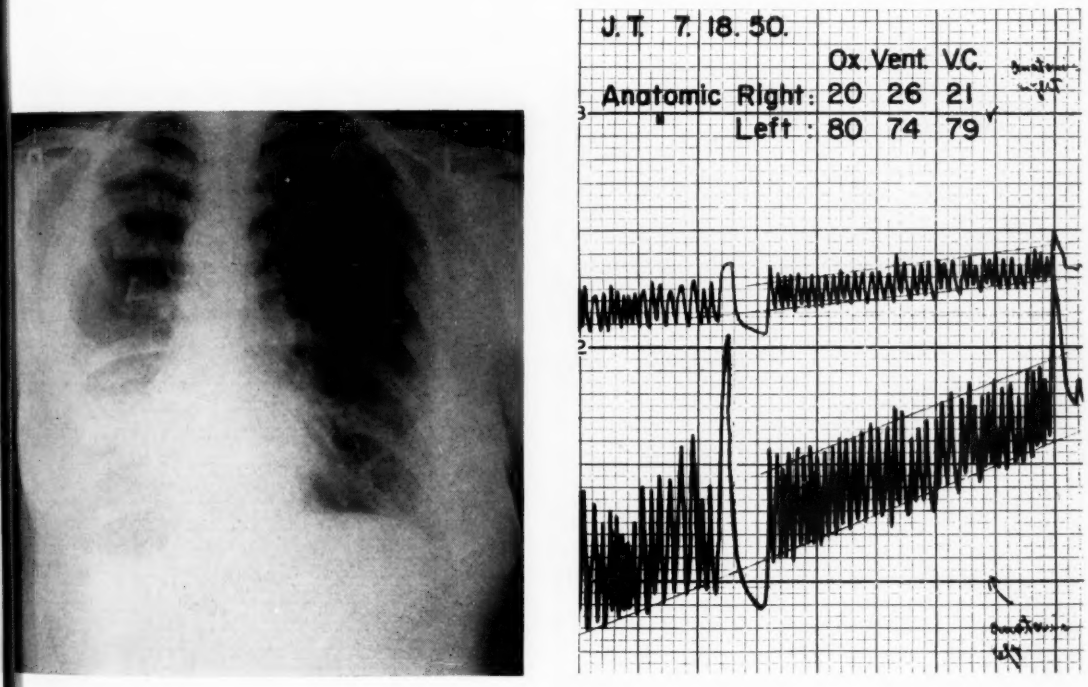
A. *B.*

Fig. 3.—Roentgenogram and bronchospirogram of J. K., a 25-year-old woman who had bronchiectasis involving both lower lobes. A left lower lobectomy proceeded uneventfully. After right lower lobectomy a bronchopleural fistula developed and the remaining right lobes collapsed. Bronchial repair and decortication of the right upper and middle lobes was planned. Since the patient had severe exertional dyspnea, it was felt that assurance should be obtained that decortication, if unsuccessful, would not aggravate the dyspnea. The MBC was only 31 liters per minute (38 per cent) and the VC was 910 ml. (28 per cent). Bronchospirometry showed that the left upper lobe remained as the only functioning lung tissue.

The MBC was 31 L. per minute (38 per cent), the walking ventilation was somewhat increased and the walking dyspnea index was 55 per cent. The VC was 910 ml. (28 per cent) and maximal expiratory velocity studies revealed no obstructive defect. Bronchospirometry showed that the right middle and upper lobes contributed nothing to function (Fig. 3).

The patient had unusually severe restrictive ventilatory insufficiency. It was felt that decortication, if unsuccessful, would not cause further loss of function and, if successful might restore this patient to a semiambulatory existence. Operability depended more on the general physical condition, and it was questioned whether a patient with so limited a pulmonary reserve would tolerate prolonged anesthesia and blood loss even if no further specific functional loss was involved.

Bronchospirometry Before Surgery in Patients Complaining of Dyspnea.—Another frequent indication for split function studies prior to surgery is exertional dyspnea. When physical examination and roentgenograms suggest that this complaint is due only to disease on the side to be treated, dyspnea is sometimes ignored. It should be remembered that even the entire loss of one lung, in the presence of an entirely normal opposite lung, does not cause troublesome shortness of breath, although exercise tolerance is limited. If dyspnea is ob-



A. *B.*

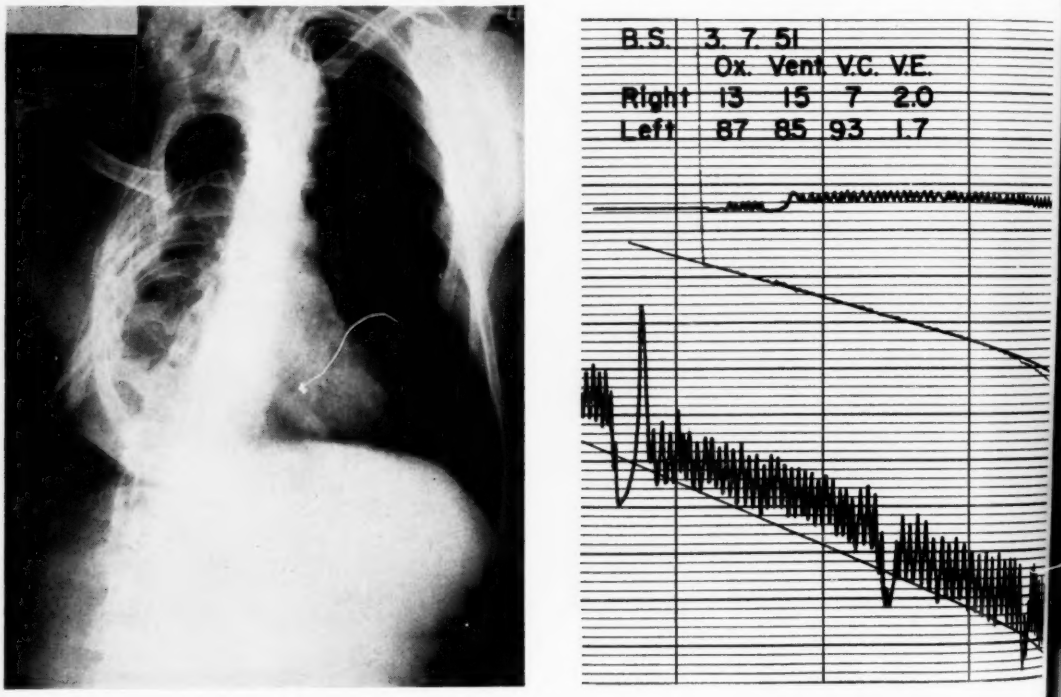
Fig. 4.—Roentgenogram and bronchospirogram of J. T., a 41-year-old woman with Kartagener's syndrome (situs inversus, bronchiectasis, and sinusitis). An empyema on the right had been drained eighteen years before and a right lower lobectomy and lingulectomy was performed six years before. Residual bronchiectasis was demonstrated in the right upper lobe. Because the patient was dyspneic studies were requested to determine the functional contribution of the remaining portions of the right lung. The MBC was 41 liters per minute (47 per cent), the VC was 1,570 ml. (55 per cent), and the air velocity index and timed capacities suggested a slight obstructive element.

served with lesser exertion some impairment of the other lung should be suspected. This suspicion led to the request for bronchospirometry in the following two cases:

J. T., a 41-year-old woman, was referred from the Lahey Clinic with a diagnosis of Kartagener's syndrome (situs inversus, bronchiectasis, and sinusitis). The patient had had a productive cough since the age of 20 years. Abdominal transposition of viscera had been observed during an appendectomy. A chronic empyema on the patient's right side had been drained nineteen years previously. Chronic hyperplastic suppurative ethmoid and maxillary sinusitis had been treated for eight years. The lower lobe and lingula had been removed four years before because of bronchiectasis and chronic pleuritis (Fig. 4). Produc-

tive cough continued and bronchograms showed bronchiectasis of the remaining portions of the lung on the right. Function studies were requested because the patient became short of breath with moderate exertion.

The MBC was 41 L. per minute (47 per cent) and the VC was 1,570 ml. (55 per cent). The air velocity index and the timed capacities revealed a moderate slowing of the maximal expiratory effort. Bronchospirometry indicated that the remaining lobe on the right was responsible for only one-fifth of the total function (Fig. 4). It was felt that a pneumonectomy would be tolerated and would probably not noticeably decrease function. The obstructive element suggested that the uninvolved lung had suffered some damage perhaps due to chronic overdistention. The resection and a thoracoplasty were performed. The postoperative course was uneventful.



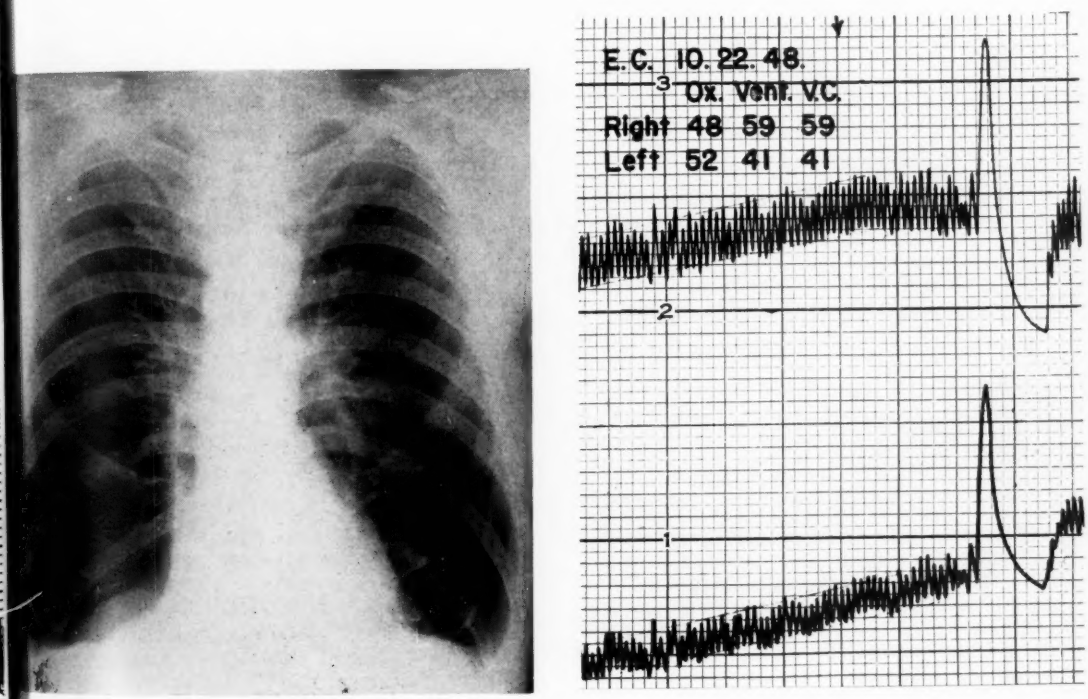
A. *B.*

Fig. 5.—Bucky roentgenogram and bronchospirogram of B. S., a 32-year-old man who had an empyema at the age of 2 years, and subsequently developed kyphoscoliosis. A right pneumonectomy was contemplated for treatment of a severe productive cough. Because of moderate exertional dyspnea studies were requested to determine the functional contribution of the right lung. The MBC was 53 liters per minute (49 per cent) and the VC was 1,680 ml. (44 per cent). The right lung was nearly functionless.

Dyspnea in the presence of apparently unilateral disease may be due to poor respiratory mechanics on the uninvolved side. This is seen occasionally after pneumonectomy or extensive thoracoplasty but is most severe in patients with kyphoscoliosis:

B. S., a 32-year-old man, was referred from the Lahey Clinic. A right-sided empyema which developed at the age of two years resulted in severe kyphoscoliosis (Fig. 5). Following drainage, fistulous tracts persisted until six years ago when the tracts were excised. A productive cough was not relieved. Bronchography traced the symptoms to the right lung

within the convexity of the spinal deformity (Fig. 5). A right pneumonectomy was contemplated. Because of moderate exertional dyspnea the operation was deferred pending examination of the functional contribution of this lung. The MBC was 53 L. per minute (49 per cent), the VC was 1,680 ml. (44 per cent), and timed capacities revealed a moderate slowing of the maximal expiratory effort. Both his residual volume and functional residual capacity were increased indicating overdistention. Since bronchospirometry showed that the right lung contributed only 13 per cent to total oxygen uptake (Fig. 5), it was thought that surgery might be performed in spite of low over-all function. A right pneumonectomy, followed by an uneventful course, caused relief of symptoms. The specimen revealed bronchiectasis, chronic pneumonitis, and fibrostenosis of the main bronchus.



A. *B.*

Fig. 6.—Roentgenogram and bronchospirogram of E. C., a 56-year-old man with exertional dyspnea of many years' duration. A large area devoid of lung markings was demonstrated at the base of the right lung. It was felt that if it could be shown that the right lung functioned less than the left the patient might benefit from resection of the bleb. The MBC was 22 liters per minute (22 per cent), the VC was 1,920 ml. (50 per cent), and the patient was unable to perform the exercise test. The air velocity index was reduced to 0.4, the residual volume occupied 55 per cent of the total lung capacity, and the intrapulmonary mixing index was markedly elevated. Operation was not advised.

Bronchospirometry Before Surgery Designed to Improve Bronchial Asthma, Pulmonary Emphysema, and Pulmonary Fibrosis.—In patients with diffuse pulmonary disease, relief is often expected by excision of an offending focus. In bronchial asthma this may be an atelectatic or bronchiectatic segment. In pulmonary fibrosis only a small area may be severely involved but may cause reflex dyspnea. Finally, in pulmonary emphysema, a giant bleb may appear to be responsible for much of the patient's respiratory difficulty:

E. C., a 56-year-old man, was referred from the U. S. Naval Hospital in Chelsea complaining of severe exertional dyspnea of many years' duration. A clinical diagnosis of pulmonary emphysema had been made. The roentgenogram showed a large area devoid of lung markings at the base of the right lung (Fig. 6). Resection of this bleb was recommended if asymmetrical function could be demonstrated.

The MBC was 22 L. per minute (22 per cent), the patient hyperventilated during the exercise test and the walking dyspnea index was 95 per cent. The VC was 1,920 ml. (50 per cent) and the air velocity index was reduced to 0.4. The residual volume of 2,700 ml. represented 55 per cent of the total lung capacity. The spirogram was characteristic of severe obstruction and the alveolar air contained 11.23 per cent nitrogen after seven minutes of pure oxygen breathing. The arterial oxygen saturation at rest was only 76 per cent and severe respiratory acidosis was demonstrated. Bronchospirometry showed essentially normal functional distribution although oxygen uptake on the right was slightly reduced (Fig. 6). Because of normal functional distribution it was felt that blebs were probably multiple and fairly symmetrically distributed. Surgery was not advised. The patient died of pulmonary insufficiency and infection shortly after closed tube drainage of the large basal bleb on the right.

In the group with diffuse disease, bronchospirometry is most useful when pulmonary fibrosis is due to radiation therapy. Damage of one lung and hemithorax may predominate though there is usually some involvement of the opposite lung which is not apparent on the roentgenogram. Productive cough, pain, and dyspnea may be due to predominantly unilateral pulmonary fibrosis and fibrothorax, or may be caused by diffuse damage to the lungs:

N. V., a 37-year-old woman, was referred from the Lahey Clinic with pulmonary fibrosis due to roentgen radiation which had been given following a right radical mastectomy four years before. Progressive fibrotic changes in the left lung were accompanied by increasing productive cough and exertional dyspnea. Bronchography revealed severe distortion and bronchiectatic changes of the tracheobronchial tree (Fig. 7).

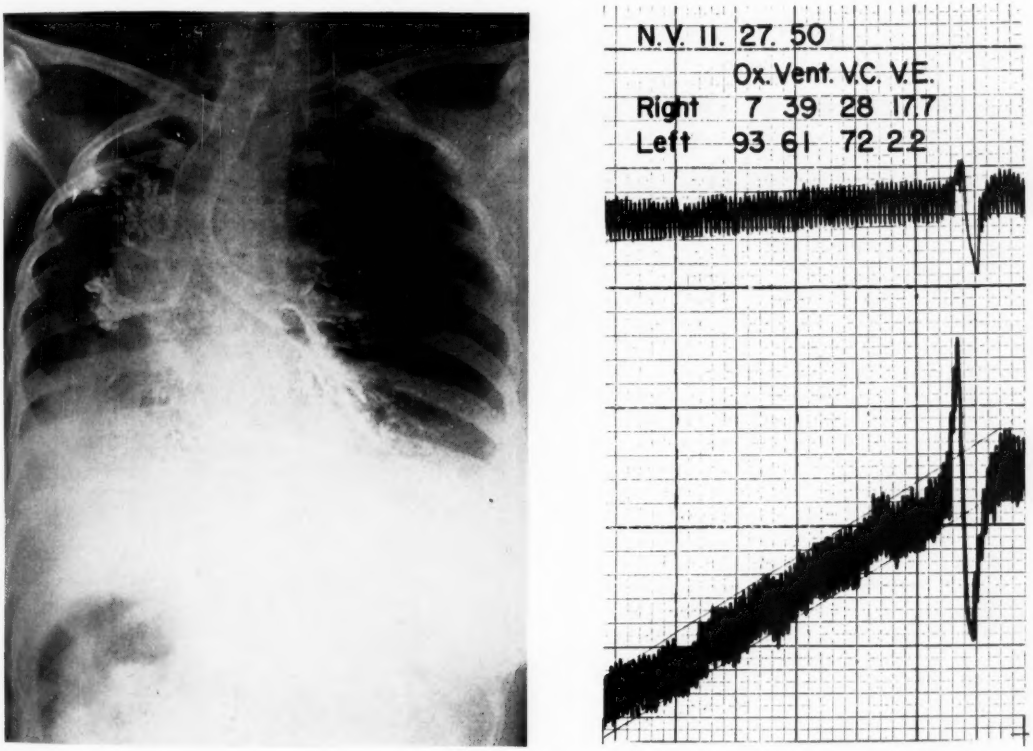
The MBC was 80 L. per minute (93 per cent), the VC was 2,080 ml. (74 per cent), exercise ventilation was normal, and the walking dyspnea index was 20 per cent. Timed capacities and the air velocity index were within normal limits, and at rest the arterial blood was 95 per cent saturated. The right lung ventilated a nearly normal volume at rest and contributed 28 per cent to the total vital capacity. However, it appeared to have lost the ability to take up oxygen (Fig. 7). It was responsible for only 7 per cent of the entire oxygen uptake.

Good over-all ventilatory capacity and the asymmetrical bronchospirogram suggested that radiation had caused predominantly unilateral damage with obstruction to right pulmonary arterial flow without greatly interfering with the lung's capacity to exchange air. It was thought that pneumonectomy would not increase the patient's functional disability. The operation was performed shortly after these studies were done. The postoperative course was uneventful, and symptoms were relieved.[9] The specimen showed marked fibrosis and chronic inflammation consistent with changes due to radiation.

The case just cited showed pulmonary damage due to radiation which was mainly confined to one lung. Unfortunately, the damage may at times be bilateral in spite of unilateral radio-opacities:

G. B., a 65-year-old woman, was referred from the Lahey Clinic four years after left radical mastectomy which had been followed by radiation therapy. She complained of exertional dyspnea and a moderately productive cough. Bronchoscopy revealed no abnormality and bronchograms showed contraction and scarring of the left upper lobe. The roentgenogram suggested unilateral involvement of the left lung (Fig. 8).

Function studies showed an MBC of 40 L. per minute (69 per cent), marked hyperventilation at rest, and during exercise and the walking dyspnea index was 50 per cent. The VC was 1,115 ml. (47 per cent). The arterial blood was only 86 per cent saturated and the difference between arterial and alveolar oxygen tension was 45 mm. at rest. Bronchospirometry showed that the lung with multiple radio-opacities was responsible for 26 per cent of the total oxygen uptake and an even larger share of resting and maximal ventilation (Fig. 8).



A. *B.*

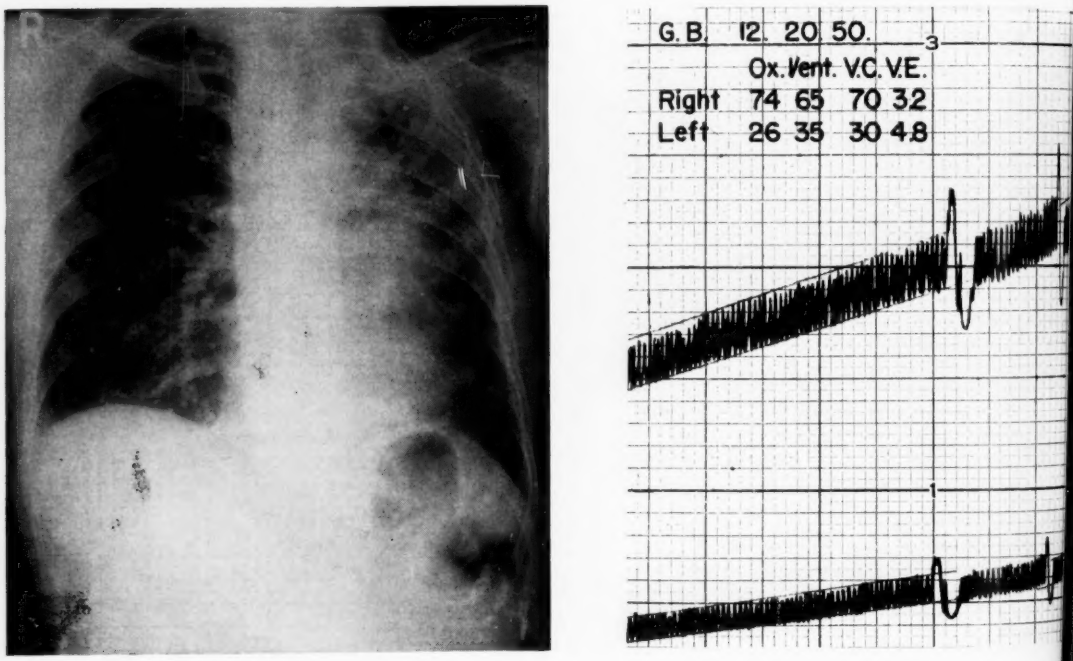
Fig. 7.—Bronchogram and bronchospirogram of N. V., a 37-year-old woman with pulmonary fibrosis due to radiation therapy after right radical mastectomy. The MBC was 80 liters per minute (93 per cent) and the VC was 2,080 ml. (74 per cent). Extensive bronchiectatic changes are shown on the right. Dyspnea and productive cough were relieved by pneumonectomy.

This patient, in striking contrast to the previous one, showed poor over-all function and a considerable functional contribution by the lung with major damage. It was felt that removal of this fairly well-functioning lung tissue would probably not relieve the productive cough and would increase exertional dyspnea. The patient was treated symptomatically and returned home after some improvement.[9]

Bronchospirometry Before Pulmonary Surgery in Patients With Bilateral Disease.—Not infrequently patients are seen at the laboratory for whom surgery is planned in spite of disease on the other side. This is often a fibrotic segment or lobe. Patients with bilateral bronchiectasis fall into this group. One of many bilateral lung cysts may become infected and require resection. Occasionally

threatening disease is seen on both sides, but the urgency for treatment appears greater on one side:

V. MacN., a 25-year-old woman, was referred from the Rhode Island State Sanatorium with a diagnosis of far-advanced pulmonary tuberculosis. On the right an unexpandable lung and hydrothorax had resulted from a therapeutic pneumothorax. The left lung had been allowed to re-expand after an extrapleural pneumothorax was shown to be ineffective (Fig. 9). Treatment for multiple cavities at the left apex was required. However, it appeared that the left lung alone was responsible for respiratory function.



A. *B.*

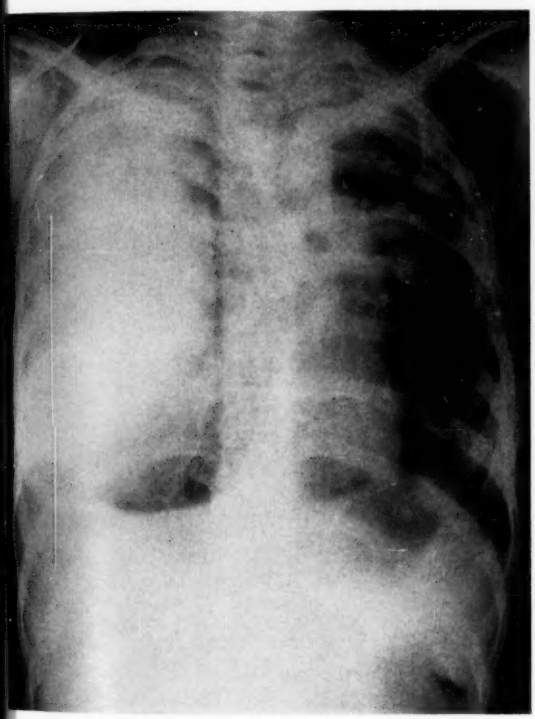
Fig. 8.—Roentgenogram and bronchospirogram of G. B., a 65-year-old woman with pulmonary fibrosis due to radiation therapy after left radical mastectomy. The MBC was 40 liters per minute (69 per cent) and the VC was 1,115 ml. (47 per cent). Marked hyperventilation was observed at rest and during exercise. The arterial blood was unsaturated at rest and a 45 mm. difference existed between the arterial and the alveolar oxygen tension. Operation was not advised.

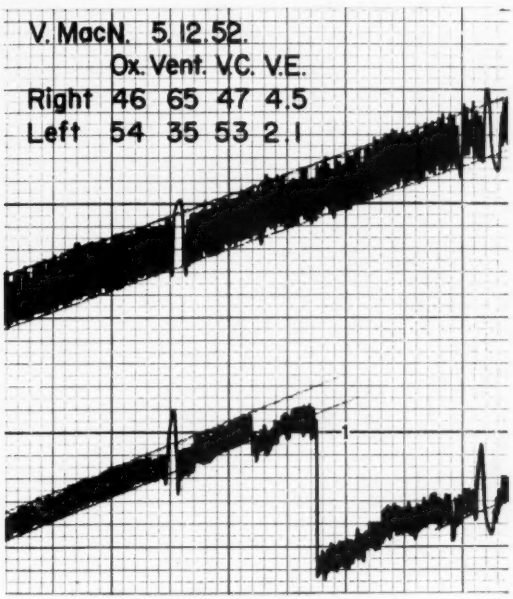
The MBC was 51 L. per minute (53 per cent), the patient hyperventilated during the exercise test, and the walking dyspnea index was 45 per cent. The VC was 1,150 ml. (35 per cent), and the timed capacities and air velocity index were normal. The bronchospirogram was surprising in view of the roentgenogram (Fig. 9). It re-emphasized the unpredictability of the effect of pleural disease from the appearance of the chest film.[3] The right lung which appeared to be almost completely collapsed was responsible for one-half of the total oxygen uptake and vital capacity and an even larger share of resting ventilation (Fig. 9).

Bronchospirometry Before Surgery in Patients With a History of Disease on the Opposite Side.—The history or previous roentgenograms often suggest that the contralateral lung had been considerably involved in the past:

F. McG., a 31-year-old diabetic man, was referred from the Rhode Island State Sanatorium with a history of pulmonary tuberculosis treated by bilateral therapeutic pneumothorax for ten years. The right lung had re-expanded well, although it had been the site of extensive disease. The left lung failed to re-expand completely and cavities persisted at the apex (Fig. 10). Function studies were requested to determine whether the patient would tolerate surgery on the left.

The MBC was 121 L. per minute (92 per cent), the walking ventilation, walking dyspnea index, timed capacities, and air velocity index were normal, and the VC was 2,850 ml. (63 per cent). The bronchospirogram again showed how misleading the roentgenogram





A. *B.*

Fig. 9.—Roentgenogram and bronchospirogram of V. MacN., a 25-year-old woman with an unexpandable lung and hydrothorax on the right and a re-expanded extrapleural pneumothorax with multiple apical tuberculous cavities on the left. The patient complained of moderate exertional dyspnea. The MBC was 51 liters per minute (53 per cent), the VC was 1,150 ml. (35 per cent), and the patient hyperventilated at rest and during exercise. The two lungs contributed almost equally to respiration.

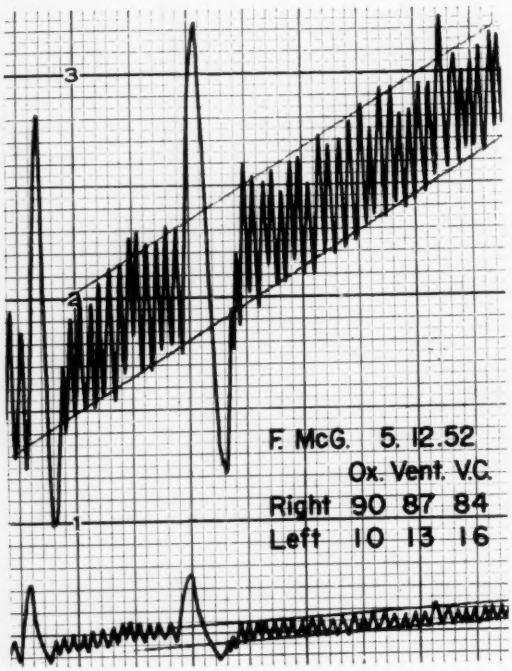
can be in the presence of pleural complications. Although some impairment of the function of the left lung was anticipated, it was not suspected that it would be almost functionless (Fig. 10).

Because of the nearly normal over-all function and the negligible contribution by the left lung, it was felt that any appropriate surgical treatment on the left side should be well tolerated.

Bronchospirometry in Preparation for a Bilateral Surgical Program.—Pulmonary function studies and bronchospirometry performed before and between

stages of planned bilateral surgery often permit a more rational approach to the surgical problem. Such evaluation frequently indicates which side should be operated on first and not infrequently suggests the type and extent of operation.

Bronchospirometry to Determine the Extent of Surgery.—Knowledge of the functional participation of the diseased lung at times suggests the extent of resection or collapse. If the functional participation is considerable, preservation of lung tissue is always advisable and especially so in patients who have disease which is prone to recur. If the lung can be shown to contribute little



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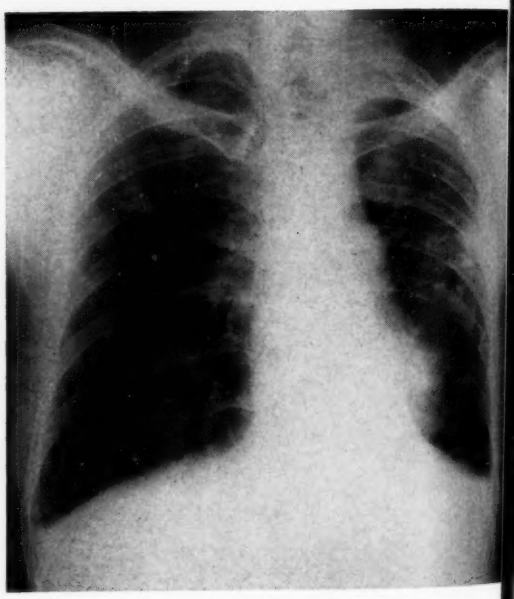


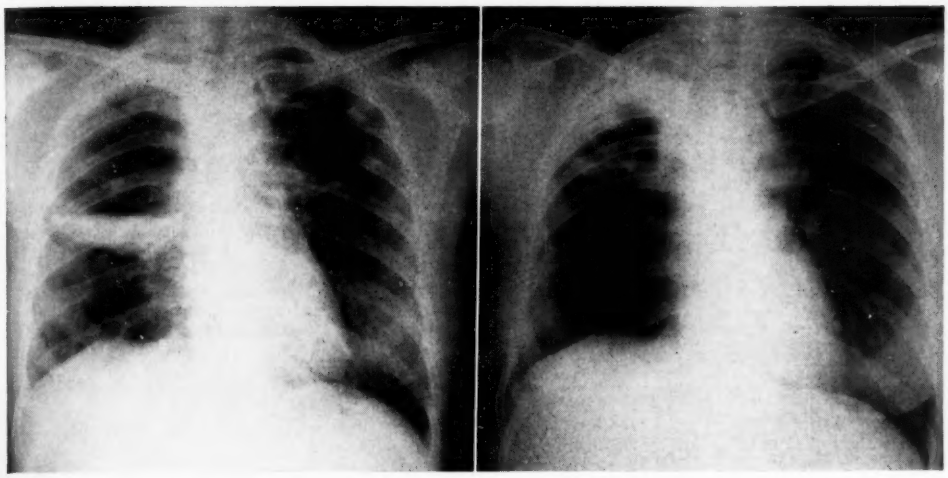
B.

Fig. 10.—Roentgenogram and bronchospirogram of F. McG., a 31-year-old diabetic man who had had bilateral therapeutic pneumothorax ten years previously. The left lung re-expanded incompletely and was the site of cavities. Further collapse treatment or resection on the left side was considered. Pulmonary function studies were requested because the contralateral lung was a re-expanded pneumothorax lung. The MBC was 121 liters per minute (92 per cent), the VC was 2,850 ml. (63 per cent), timed capacities and air velocity index were normal, as was the breathing reserve at rest and during exercise.

to over-all function, it may at times be advisable to remove the entire lung rather than take the risk of leaving behind diseased tissue:

I. K., a 40-year-old woman, was referred from the Channing Home for Tuberculosis. A giant cavity occupied the upper half of the right lung (Fig. 11).

Although the patient did not complain of exertional dyspnea, over-all function was poor (Table II). The air velocity index and timed capacities suggested an obstructive defect. Bronchospirometry at this time showed that the right lung was responsible for about one-fifth of the total function (Fig. 11).



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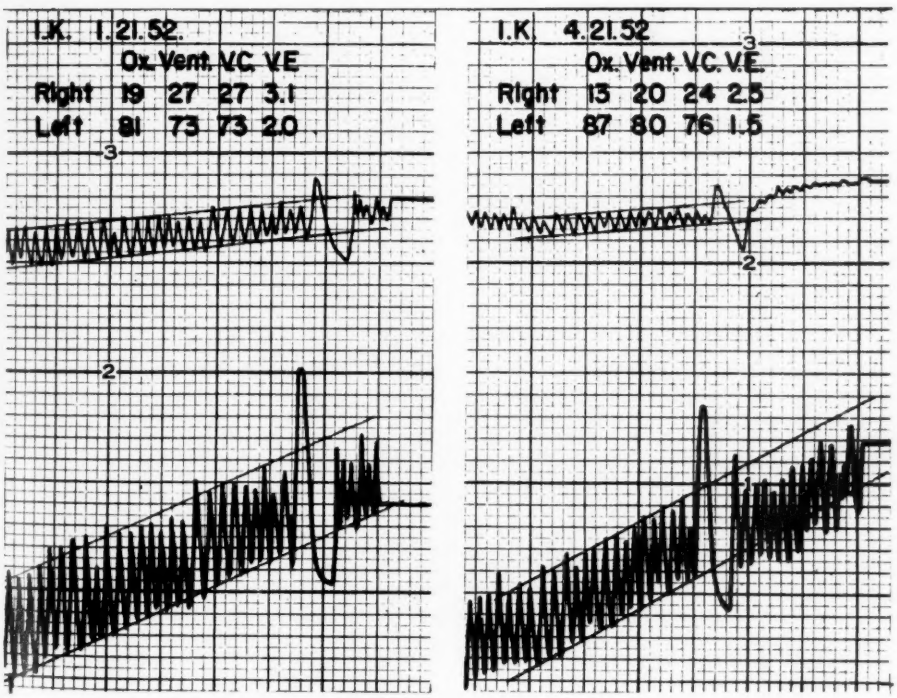


B.

Fig. 11.—Roentgenograms and bronchospirograms of I. K., a 40-year-old woman with a giant tuberculous cavity at the apex of the right lung. The first film and tracing were obtained shortly after admission. Moderate dyspnea was noted on mild exercise. Function studies are shown in Table II. The patient was seen again four months later after treatment with streptomycin and PAS. The size of the cavity had decreased, but repeated bronchoscopies showed progressive obstruction of the right upper and intermediary bronchi. Pulmonary function studies remained unchanged (Table II) except that timed capacities showed a further slowing of the maximal expiratory effort. The second bronchospirogram showed further diminution of function on the right, presumably due to progressive obstruction.

In view of the poor over-all function there was some reluctance to remove the entire right lung. Lobectomy did not seem advisable because of diffuse disease in the remaining portions of the lung. The patient was treated with streptomycin and PAS. The cavity diminished in size (Fig. 11), but repeated bronchoscopies revealed a progressive stenosis of the right upper lobe bronchus with angulation and narrowing of the right lower and middle lobe bronchi. The patient was returned for studies three months later to determine whether the performance of the right lung had improved and to ascertain if right upper lobectomy would be the procedure of choice.

TABLE II. PULMONARY FUNCTION STUDIES OF PATIENT I. K. WITH INCREASING TUBERCULOUS BRONCHOSTENOSIS

EXAMINATION	PREDICTED NORMAL	FIVE MONTHS PREOPERATIVE	TWO MONTHS PREOPERATIVE
Maximum breathing capacity, L./min.	80	33	33
Vital capacity, ml.	2,760	1,550	1,550
Walking ventilation, L./min.	15	13	13
Walking dyspnea index, %	19	40	40
Air velocity index	1.0	0.7	0.7
Timed vital capacities, % of total VC			
First, first two, and first three seconds	75-90-100	58-73-77	49-65-72
Bronchospirometry, *right* lung			
Oxygen uptake, % of total	55	19	13
Resting ventilation, % of total	55	27	20
Vital capacity, % of total	55	27	24

The MBC and VC were entirely unchanged (Table II). Timed capacities indicated a further slowing of the maximal expiratory effort. Bronchospirometry showed that, although vital capacity had decreased only slightly, ventilation on the right was reduced by seven per cent and that the lung now contributed only 13 per cent of the total oxygen uptake. The appearance of the spirogram also suggested progressive airway obstruction (Fig. 11).

The lesser participation of the right lung was attributed to increasing bronchial obstruction. Preservation of the diseased right lower lobe appeared to be of little functional advantage. A right penumonectomy, two months later, was followed by an uneventful postoperative course.

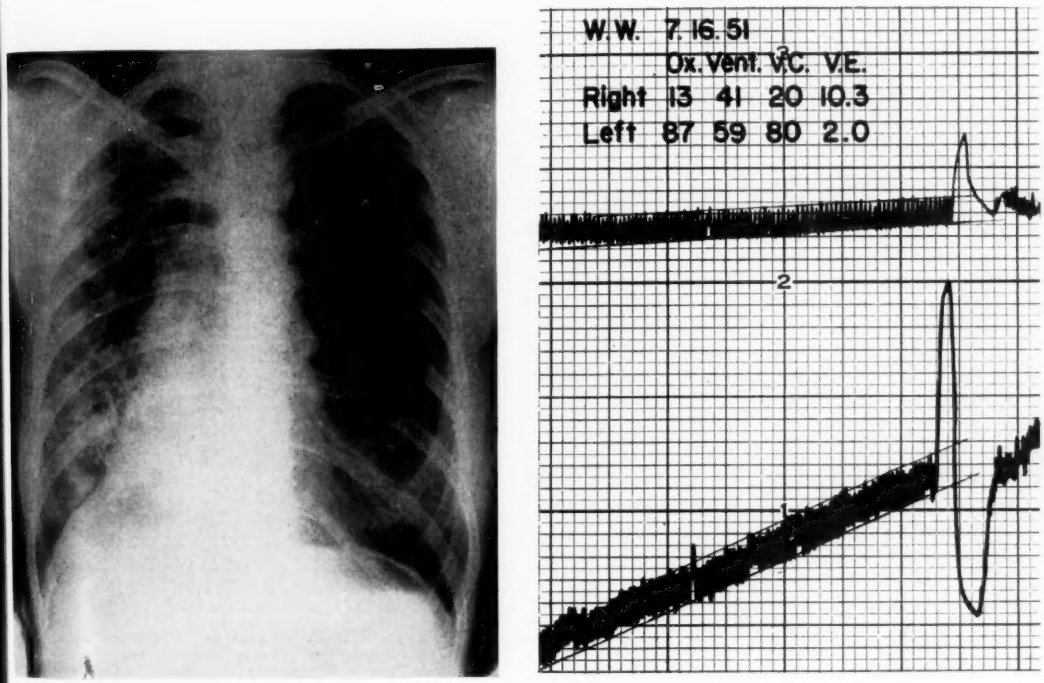
Bronchospirometry to Determine Asymmetry of Function in Apparently Unilateral Disease.—On a number of occasions we were asked to determine the functional distribution in patients who, according to the roentgenogram, had involvement of one lung only. This problem often arises in diseases which usually have a symmetrical distribution. In sarcoidosis or the pneumonoconioses the studies were chiefly of theoretical interest. However, when the disease was of a type amenable to surgery, bronchospirometric findings had considerable practical interest. The cases with pulmonary fibrosis due to radiation (Figs. 7 and 8) might well have been included here. The request for bronchospirometry in this connection occasionally arose in patients with cystic disease of the lung or with congenital bronchiectasis:

W. W., a 58-year-old woman, was referred by the Brockton Hospital with a diagnosis of congenital bronchiectasis involving the right lung (Fig. 12). The patient had been subject to recurrent pneumonia and had had a severe productive cough for as long as she could remember. A pneumonectomy was deferred because of the patient's age and some exertional dyspnea.

The MBC was 56 L. per minute (78 per cent), the VC was 2,490 ml. (93 per cent), exercise ventilation was normal, and the walking dyspnea index was slightly elevated. Timed capacities, air velocity index, and spirogram indicated slight slowing of the maximal expiratory

effort. The residual volume was 950 ml., which was normal in relation to the total lung capacity. The intrapulmonary mixing index of 1.25 per cent nitrogen was within normal limits. The results of bronchospirometry were unusual. The right lung ventilated almost normally at rest but contributed only 13 per cent to the total oxygen uptake and 20 per cent to the vital capacity (Fig. 12). The extensive bronchiectatic process had damaged the pulmonary vascular bed and limited maximal expansion without affecting ventilation at rest. The findings were reminiscent of those in the patient with bronchiectatic changes due to radiation therapy (Fig. 7). The relative hyperventilation of the right lung was expressed by a ventilation equivalent of 10.3 on the right and only 2.0 on the left.

Because the studies suggested the function of the good lung had not been impaired, it was felt that pneumonectomy was justified.



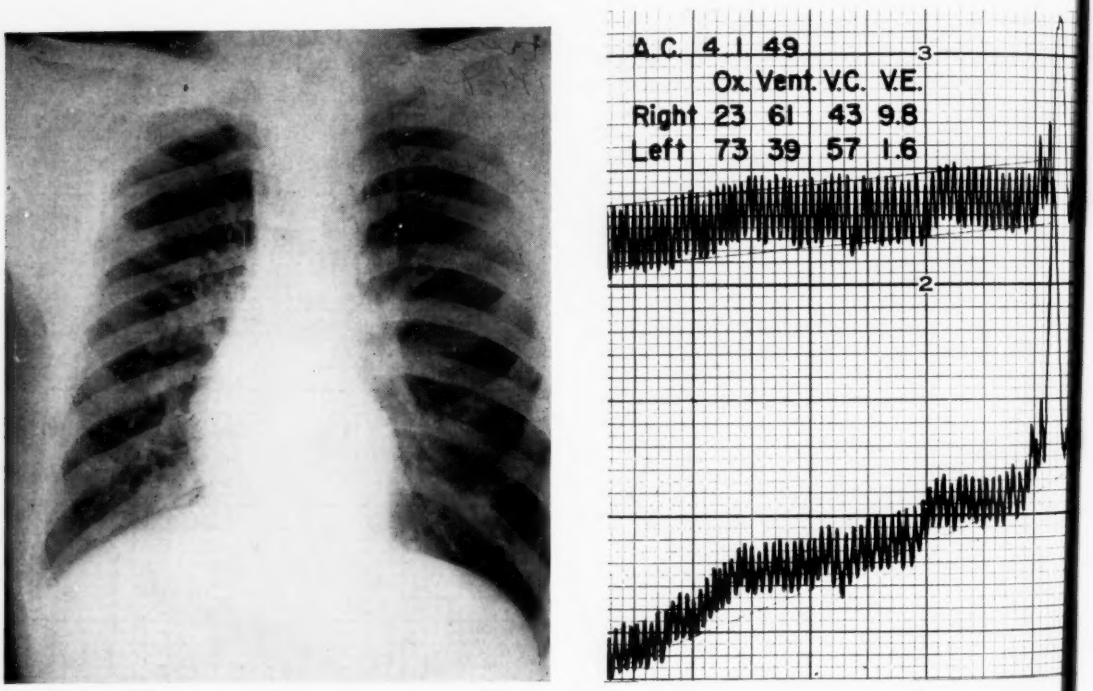
A. *B.*

Fig. 12.—Roentgenogram and bronchospirogram of W. W., a 58-year-old woman with congenital bronchiectasis of the right lung. Pneumonectomy was deferred because of exertional dyspnea. The MBC was 56 liters per minute (78 per cent), the VC was 2,490 ml. (93 per cent), and timed capacities and the air velocity index revealed moderate slowing of the maximal expiratory effort. The residual volume occupied 28 per cent of the total lung capacity, and the intrapulmonary mixing index was normal

The results of physical examination, roentgenography, and fluoroscopy may be most deceptive in diseases where normal ventilation is maintained in a lung with severely diminished blood flow. In congenital cystic disease of the lung some oxygen uptake is nearly always preserved but the difference between ventilation and perfusion may nevertheless be striking:

A. C., a 19-year-old boy, was referred from the Fourth Medical Service of the Boston City Hospital with a diagnosis of congenital cystic disease of the right lung. Multiple cysts could be seen on the plain chest film and the lung appeared well aerated (Fig. 13).

The MBC was 118 L. per minute (92 per cent), exercise ventilation and walking dyspnea index were somewhat elevated, the VC was 3,250 ml. (94 per cent), and timed vital capacities and the air velocity index were normal. Bronchospirometry illustrated the discrepancy between ventilation and perfusion (Fig. 13). The right lung was responsible for only 23 per cent of the total oxygen uptake, but participation in ventilation and vital capacity were unaffected. The ventilation equivalent was six times larger on the right than on the left. The arterial oxygen saturation during bronchospirometry was 96 per cent breathing ambient air and 101 per cent while breathing an oxygen-enriched mixture. The diminished oxygen uptake was

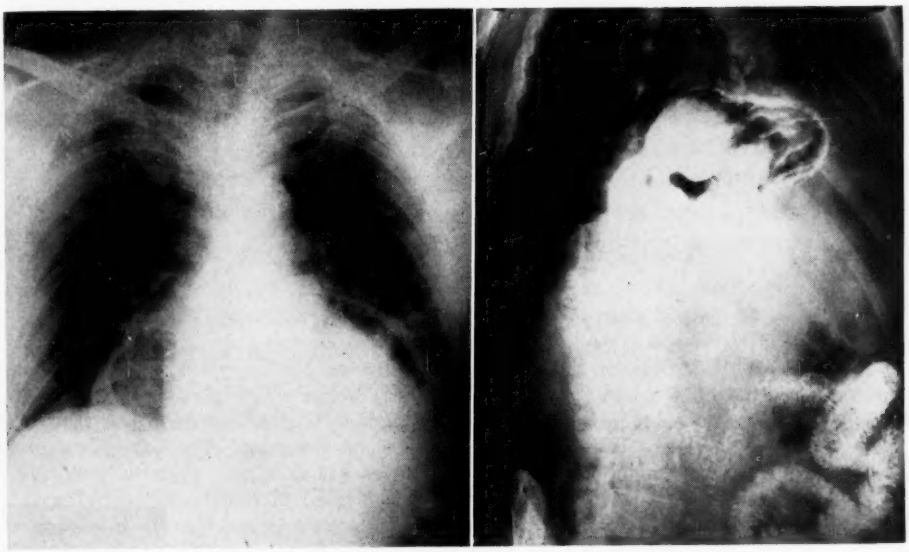


A. *B.*

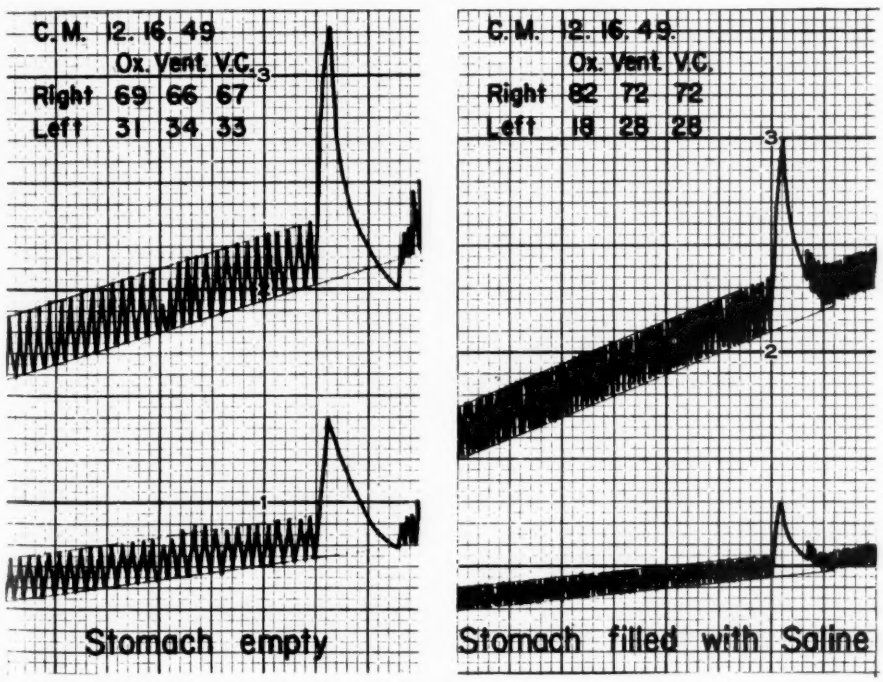
Fig. 13.—Roentgenogram and bronchospirogram of A. C., a 19-year-old boy with congenital cystic disease of the right lung. Fluoroscopy suggested normal air exchange on the right. Studies were requested to determine how much the right lung contributed to total pulmonary function. The MBC was 118 liters per minute (92 per cent) and the VC was 3,250 ml. (94 per cent). Residual volume and intrapulmonary mixing studies were normal.

therefore due to poor arterial perfusion rather than to poor oxygenation. Because the patient was free of symptoms it was decided to defer radical treatment to a time when infection of the cysts might cause difficulties.

Bronchospirometry to Evaluate the Success of "Reconstructive" Chest Surgery.—The results of operations intended to improve the cardiopulmonary status are not easily quantitated. The severity of dyspnea, fatigue, and limitation of activity are often altered by the psychic impact of surgery and the encouragement following operation. An extensive series of cardiorespiratory measurements are now being made before and after such procedures. It is hoped that these studies will provide surgeons with a quantitative evaluation of the end-results and may eventually help to establish a set of indications for such operations.



A.



B.

Fig. 14.—Roentgenograms and bronchospirograms of C. M., a 57-year-old man with a diaphragmatic hernia. He complained of severe dyspnea in the supine position and after meals. Studies were requested to determine if a left transthoracic approach would be advisable and to show if the herniation actually encroached on the lung. The results of ventilatory studies before and after a large meal are summarized in Table III. The first bronchospirogram was obtained in the supine position with the patient's stomach empty. The second tracing was recorded some minutes later after 1,500 ml. of saline solution had been injected in the stomach through a rubber tube.

Bronchospirometry enters the picture when "reconstructive" operations are performed to improve the function of a previously disabled lung (Table I). Pulmonary decortication following trauma, empyema, pleural effusion, and for the treatment of unexpandable pneumothorax is the most frequently encountered "reconstructive" operation. The functional results of this procedure have been reviewed by others and by our own group.[1, 10]

The last patient is shown because he illustrates the need for an unusual type of pulmonary functional reconstructive surgery which does not involve the lungs directly:

C. M., a 57-year-old retired wrestler, was referred by the Thoracic Surgery Service of the Boston City Hospital. He complained of dyspnea on lying down and dyspnea after meals. He had had recurrent hematemesis and melena for ten years, and postprandial epigastric distress for the past five years. Two years before this admission an attempt was made by another service to reduce a large diaphragmatic herniation which was thought to have been due to a congenital defect of the foramen of Morgagni. The left phrenic nerve was interrupted and the anterior portion of the defect was repaired. Posteriorly the defect was too large for closure (Fig. 14). The patient continued to bleed occasionally but complained more and more of severe dyspnea following meals and was unable to assume the supine position without becoming short of breath. Studies were requested to determine whether over-all function and respiratory distribution would permit a renewed left transthoracic approach. The surgeons also wished to ascertain whether dyspnea was due to intrapulmonary disease or was caused entirely by pulmonary compression due to the herniation.

Function was studied before and after a heavy meal (Table III). A very low MBC, a low air velocity index and timed capacities, a residual volume which occupied 62 per cent of the total lung capacity, and a marked elevation of the intrapulmonary mixing index established a diagnosis of pulmonary emphysema. The studies further showed that ventilation was more severely impaired after eating (Table III). Because of the underlying severe emphysema a further reduction, although quantitatively small, was severe enough to cause a marked increase of symptoms.

On another occasion a stomach tube was passed after which the trachea was intubated in the usual way for bronchospirometry. The patient was placed in the supine position and the first record was obtained with the stomach empty (Fig. 14). The stomach was then filled rapidly with 1,500 ml. of saline solution through the tube and the bronchospirometric

TABLE III. PULMONARY FUNCTION STUDIES OF PATIENT C. M. WITH A DIAPHRAGMATIC HERNIA

DETERMINATION	PREDICTED NORMAL		BEFORE MEAL		AFTER MEAL	
Maximum breathing capacity, L./min.	108		41		31	
Walking ventilation, L./min.	18		25		27	
Walking dyspnea index, %	17		61		87	
Air velocity index	1.0		0.7		0.6	
Vital capacity, ml.	3,520		1,910		1,600	
Residual volume, ml.	1,530		3,090		2,800	
Total lung capacity, ml.	5,050		5,000		4,400	
R.V./T.L.C. ratio, %	30		62		64	
Intrapulmonary mixing index, % N_2	<1.50		7.42		6.48	
	PREDICTED NORMAL		BEFORE GASTRIC INFUSION		AFTER GASTRIC INFUSION	
	RIGHT	LEFT	RIGHT	LEFT	RIGHT	LEFT
Bronchospirometry						
Oxygen uptake, % of total	55	45	69	31	82	18
Resting ventilation, % of total	55	45	66	34	72	28
Vital capacity, % of total	55	45	67	33	72	28
Ventilation equivalent, L./100 c.c.	2.2	2.2	2.0	2.3	2.5	4.4

examination was repeated (Fig. 14). The effect of this infusion was dramatic. The tidal volume was somewhat decreased on both sides and the respiratory rate nearly doubled. In addition, the left lung which had formerly contributed 31 per cent of the total oxygen uptake and 33 per cent of the total vital capacity, now contributed only 18 per cent to oxygen uptake and 28 per cent to vital capacity. These studies indicated that over-all ventilation was markedly embarrassed by a full stomach and that the herniation compressed the left lung to a greater degree than the right (Table III).

SUMMARY

The indications for bronchospirometry in a series of 1,089 examinations are listed. Although 71 per cent of all tests were performed primarily for "investigational" purposes, it was felt that the procedure will eventually find its widest application in the field of clinical evaluation.

Fourteen patients from a series of 311, referred for evaluation prior to surgery, were selected for individual presentation. They illustrate the commonly encountered indications.

The technical assistance of Miss D. M. Donnelly and Miss S. S. Smith was invaluable.

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LABORATORY METHODS

LABORATORY STUDIES OF HISTOPLASMA CAPSULATUM

III. Efficiency of the Flotation Method in Isolation of Histoplasma Capsulatum from Soil

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ONE of the most interesting facets of the epidemiology of coccidioidomycosis and histoplasmosis has been the reported sharp geographic localization of these diseases—coccidioidomycosis to the southwest and histoplasmosis to the Midwest. These geographic limitations may be explained by climatic conditions, soil types, or some characteristics peculiar to the two fungi. It has been assumed that the etiological agents of the diseases exist saprophytically in nature. Confirmation of this assumption rests on the isolation of the organisms from soil or from other environmental foci. A significant point in this hypothesis is the relative infrequency with which either organism has been isolated from soil sources in the endemic areas. Emmons and Ashburn,[1] Stewart and Meyer,[2] and Davis, Smith, and Smith[3] have reported the isolation of *Coccidioides* from soil or sand. Emmons and Ashburn provide the only report on the quantitative aspects. They were successful in isolating this fungus on five occasions from 150 soil samples, that is, only 3.3 per cent of their tested soil samples proved to be positive.

A similar situation occurred with *Histoplasma*. Emmons[4] has reported two positive soils from 387 samples tested, or 0.5 per cent.† Likewise, Zeidberg and associates[6] have reported 11 isolations from 299 soil samples, or 3.7 per cent.

The reason for relatively infrequent soil isolations may have been either absence of the organism in the samples or inadequate methods of testing. To test the latter possibility, a series of experiments was designed to evaluate the efficiency of the flotation method in isolating *Histoplasma capsulatum* from soil samples.

The flotation method has been employed extensively in isolating pathogenic organisms from natural sources. Usually investigators modified the method described by Stewart and Meyer[2] in their studies of *Coccidioides immitis*. This method consists of attempts to concentrate the fungus spores in the supernatant layers obtained after shaking the soil in physiological or hypertonic salt solutions. The supernatant fluid is then used for the inoculation of culture plates

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†Emmons in a later pulication reported twenty-eight additional isolations of *Histoplasma capsulatum* from soil; however, no mention was made of the number of soils studied.[5]

or of mice. The key to the flotation method is the supposed concentration of the fungus spores in the superficial layers of the suspension. Since this concentration has never been established experimentally, as far as can be ascertained, the purpose of these experiments was to determine where the spores of *H. capsulatum* might be found in the suspension. Do the spores float in the supernatant fluid; do they remain adherent to the heavy soil particles; can they be concentrated by some variation of the accepted flotation method or by some new modification?

MATERIALS AND METHODS

These experiments represent attempts to recover *Histoplasma* spores following their addition to sterile soil. Since an attempt was made to account for all of the spores added to the soil, the experiments serve not only to evaluate the flotation method, but also to locate the majority of the spores in the soil.

Two experiments were performed using sterilized soil. The first was freshly inoculated with the spores of *Histoplasma;* the second had been inoculated fourteen months previously, and growth was evident.

1. *Experiments With Freshly Inoculated Soil.*—Four strains of *H. capsulatum* from the stock collection in Kansas City were selected. The isolates were from proved human cases (Mitchell, Ellis, Chick, and Benner) and were inoculated onto a modified Sabouraud's agar containing 1 per cent dextrose (hereafter designated as Sabouraud's agar) in 250 ml. Erlenmeyer flasks. After incubation at room temperature for twenty-one days, the cultures were used as the source of the inoculum in the investigations. The inoculum from each isolate was scraped from the flask with an L-shaped loop, transferred to a sterile glass tissue grinder, and macerated. This procedure proved highly satisfactory in producing a homogeneous inoculum. Prior to adding the inoculum to the designated cylinders, a spore count of each isolate was made using a hemacytometer. Viability counts were also made by plating 1 ml. of the suspension onto each of 10 Sabouraud's agar plates. Counts were made of the developing colonies on all the plates at ten, fourteen, and twenty-six days.

Loam soil collected from the hospital grounds of the University of Kansas Medical Center was sterilized in flasks by autoclaving for two hours at 124° C. and 18 pounds pressure on two consecutive days. Ten milliliters of sterile soil was dispersed in each of a series of sterile 100 ml. volumetric cylinders. After determining the approximate number of spores per milliliter by hemacytometer count, 1 ml. of the inoculum was added to the soil in the designated cylinders. Sterile physiological saline was added to each cylinder to make a total volume of 100 milliliters. The cylinders were shaken vigorously for five minutes, then permitted to stand undisturbed for sixty minutes. Using sterile pipettes, 10 ml. aliquots of the supernate were removed from various regions in each of the cylinders. Ten plates of Sabouraud's agar containing 40 units streptomycin and 20 units penicillin per ml. of media were inoculated with the suspension from each of the areas of the supernate. The remaining supernate (usually 35 to 40 ml.) not used in the above experiments was transferred to sterile centrifuge tubes and centrifuged at 2,500 r.p.m. for thirty minutes. The centrifuged supernate was discarded with the exception of the lower 10 ml. which was cultured in the same manner as the other aliquots of the supernate. Finally the sedimented soil was diluted one-tenth in sterile saline. One milliliter of the dilution was plated onto each of ten plates of the medium. The growth of the fungus was determined after fifteen days.

Thus a search was made in five areas for spores; the top of the supernatant fluid or the superficial layer; the middle of the supernate; the bottom of the supernate; the sediment secured by centrifuging the remaining supernate and, finally, the sedimented soil itself.

2. *Experiments Employing Soil Inoculated Fourteen Months Previously.*—The data obtained from the above experiments suggested an investigation to determine if these findings could be substantiated with soil cultures. The nature of the soil cultures seems more nearly to simulate natural conditions for the growth of the fungus than did the freshly inoculated

soil used in the above experiments. Fortunately, soil cultures which had been inoculated 14 months previously were available.

Earlier research had established the optimal temperature and humidity requirements for the growth and development of *H. capsulatum*.[7] These results suggested that precautionary measures were necessary to assure growth and development. Therefore, 300 ml. of distilled water was dispersed into a series of one-half gallon wide-mouthed glass jars. Into each jar was placed a 250 ml. Erlenmeyer flask containing 100 ml. of sifted or ground loam soil. The Erlenmeyer flasks were not plugged but the lids of the jars were screwed down lightly, and the experimental setup autoclaved on two consecutive days for two hours at 124° C. and 18 pounds pressure. After the jars and the soil were cool, 5 drops of the inoculum were dispersed into each of the flasks. The source of the inoculum was a 21-day-old culture of the Mitchell isolate. The inoculum was macerated and transferred to the sterile soil in an inoculating chamber to reduce the chances of contamination.

Periodic examinations were made to determine if growth of *H. capsulatum* had occurred in the flasks. Within a period of three weeks, a scanty, prostrate, grayish-white mycelium developed on the surface of some of the soil. To ascertain whether the growth was *H. capsulatum*, a small sample was removed from a designated number of flasks and examined microscopically. The remainder of the jars were undisturbed until the beginning of these experiments.

The procedures followed in determining the efficiency of the flotation method in recovering the fungus from soil cultures were similar to those previously described. The only difference was the addition of actidione* to the Sabouraud's agar plates in concentrations of 0.1, 1.0, and 1.5 mg. per milliliter of medium.

RESULTS

1. *With Freshly Inoculated Soil.*—Five areas in the experimental setup were examined as possible foci of the added spores. These areas are designated as top, middle, and bottom of the supernatant fluid, the sediment of the centrifuged supernate, and the sedimented soil. The results of these studies are shown in Table I.

TABLE I. EFFICIENCY OF THE FLOTATION METHOD. NUMBER AND PERCENTAGE OF SPORES OF *H. capsulatum* RECOVERED FROM DIFFERENT LAYERS OF A SOIL SALINE SUSPENSION AFTER FIVE MINUTES' SHAKING AND ONE HOUR SEDIMENTATION. EXPERIMENTS CONDUCTED WITH FOUR ISOLATES FROM HUMAN CASES. SPORE SUSPENSION ADDED TO SOIL IMMEDIATELY BEFORE EXPERIMENT. COLONIES COUNTED AT FIFTEEN DAYS

SOURCE OF SAMPLE	MITCHELL		ELLIS		CHICK		BENNER		TOTAL	
	NO. OF COLONIES	PER CENT OF TOTAL	NO.	%	NO.	%	NO.	%	NO.	%
Top of supernate	3	0.14	9	0.07	12	0.15	36	0.16	60	0.13
Middle of supernate	3	0.14	10	0.08	11	0.13	11	0.05	35	0.08
Bottom of supernate	163	7.87	652	4.98	612	7.40	450	2.00	1,877	4.08
Centrifuged supernate	62	3.00	1,550	11.84	711	8.60	483	2.14	2,806	6.11
Sedimented soil	1,840	88.85	10,870	83.03	6,920	83.72	21,550	95.65	41,180	89.60
Total	2,071	100.00	13,091	100.00	8,266	100.00	22,530	100.00	45,958	100.00

The number of spores reported represents the number of colonies which developed on ten plates of Sabouraud's agar. As in other bacteriological and mycological studies, the assumption was made that each colony developed from a single spore.

*Supplied by Eli Lilly and Co.

Examination of Table I reveals that the results obtained with the four strains of *Histoplasma* are roughly the same. The percentages of spores in the five different sections, however, varied greatly. The most striking result is the failure of the spores to appear in the top of the supernate. The average for the four strains was only 0.13 per cent of the total spores. In other words, less than 1 per cent of the spores floated on or near the top of the suspension. There is, therefore, a deficiency rather than a concentration of spores in this area.

The inoculum removed from the middle of the supernate contained an average of only 0.08 per cent of the spores. The samples from the bottom of the supernatant fluid gave somewhat better yields. The average for the four strains was 4 per cent. It varied from about 2 per cent with the Benner strain to 7.87 per cent with the Mitchell strain.

The percentage of spores found in the bottom of the centrifuged supernatant fluid was 6.1 per cent of the total. While this was higher than the three areas previously examined, it still represents no great concentration of the spores.

Most of the spores were found in the sedimented soil at the bottom of the graduated cylinder. The average for the four strains was over 89 per cent with a variation of from 83 per cent for the Ellis strain to over 95 per cent for the Benner strain. Shaking and extraction of the soil with the salt solution had evidently not removed more than a small portion of the spores. In all the extracting fluid only a little over 10 per cent of the spores were found.

When the total number of spores recovered in each strain was compared with the number of viable spores added to the experimental cylinders, it was possible to calculate the efficiency of the experimental procedures. These tabulations are shown in Table II.

TABLE II. EFFICIENCY OF THE FLOTATION METHOD. RATIO OF NUMBER OF SPORES INOCULATED INTO SOIL TO NUMBER RECOVERED BY EXPERIMENTAL PROCEDURES

	MITCHELL	ELLIS	CHICK	BENNER	TOTAL
Number recovered by culture	2,071	13,091	8,266	22,530	45,958
Number of viable spores inoculated	6,000	16,000	11,200	29,000	62,200
Per cent of recovery	34.52	81.82	73.80	77.69	73.89

The smallest percentage of recovery was from the Mitchell strain where slightly less than 35 per cent of the spores were recovered. The greatest recovery, 82 per cent, was from the Ellis strain. Comparison of the total number of spores inoculated in all of the experiments and the total number recovered shows the method to be 74 per cent efficient.

The results of the above investigations strongly suggest that the area most likely to yield *H. capsulatum* from the soil is the sedimented soil itself. In these experiments, the authors were aware that the methods employed were artificial and did not necessarily simulate natural conditions. The use of inoculum previously grown on an artificial medium introduced the possibility that the specific gravity of the spores may have exceeded that of the spores produced in nature. This may explain why the greatest number of spores was recovered from the sedimented samples of soil.

It is quite apparent, therefore, that the flotation method of concentrating *Histoplasma* spores is not efficient.

2. *With Soil Inoculated Fourteen Months Previously.*—The results of the application of similar methods to soil cultures of *H. capsulatum* are shown in Table III.

TABLE III. EFFICIENCY OF THE FLOTATION METHOD. NUMBER AND PERCENTAGE OF SPORES OF *H. capsulatum* RECOVERED FROM DIFFERENT LAYERS OF A SOIL SALINE SUSPENSION AFTER FIVE MINUTES' SHAKING AND ONE HOUR SEDIMENTATION. COMPARISON OF RESULTS WHEN ACTIDIONE WAS ADDED TO THE CULTURE PLATES IN VARIOUS CONCENTRATIONS. EXPERIMENTS CONDUCTED WITH SOIL IN WHICH THE MITCHELL STRAIN OF *H. capsulatum* HAD BEEN GROWING FOR FOURTEEN MONTHS. COLONIES COUNTED AT FIFTEEN DAYS

SOURCE OF SAMPLE	MG. OF ACTIDIONE PER ML. OF MEDIA						TOTAL	
	0.1		1.0		1.5		NO. OF COLONIES	PER CENT OF TOTAL
	NO.	%	NO.	%	NO.	%		
Top of supernate	73	0.09	130	0.16	143	0.11	346	0.12
Middle of supernate	23	0.03	14	0.02	34	0.03	71	0.02
Bottom of supernate	120	0.15	69	0.08	121	0.10	310	0.11
Centrifuged supernate	154	0.19	167	0.20	224	0.17	545	0.19
Sedimented soil	79,600	99.54	83,200	99.54	126,800	99.59	289,600	99.56
Total	79,970	100.00	83,580	100.00	127,322	100.00	290,872	100.00

It is apparent from study of Table III that the results secured with the soil cultures were similar to those secured with the freshly inoculated soils. Much less than 1 per cent of the spores were found in the top of the supernatant fluid, and similar low percentages in the other fractions. Almost all (99.6 per cent) of the spores were found in the sedimented soil layer. The most striking difference in the results of this experiment compared to the earlier experiment is in the failure of a significant percentage of the spores to be found in the bottom of the supernate or in the centrifuged supernatant layer. It thus appears that with soil cultures the spores are even more intimately bound with the soil particles than with freshly inoculated soil.

Another point evident from Table III is that actidione in concentrations up to 1.5 mg. per milliliter of media does not significantly inhibit the growth of *H. capsulatum.* The largest spore count occurred with the greatest concentration of this antibiotic.

A number of subsequent attempts were made to separate the spores from the sedimented soil particles by variation of the extraction method. After the first shaking, the suspension was allowed to sediment and the supernatant fluid was removed, centrifuged, and cultured. Fresh saline was then added and the process repeated. No success was obtained in spite of repeated attempts employing varying periods of shaking and sedimentation up to two hours. The largest percentage of the spores was always found in the sediment.

3. *Intermediate Method.*—In view of the above findings, efforts were directed toward developing improved methods for isolating *Histoplasma* from soil. Our first, or so-called *intermediate method,* was as follows: Ten ml. of soil were placed in a graduated cylinder and saline solution added to 100 ml. After 5 minutes' shaking and 60 minutes' standing, the supernatant fluid was decanted and centrifuged. The sediments were combined in a 7 ml. volume

and streptomycin and penicillin added to make a concentration of 8,000 units of each per ml. One ml. of this mixture was then injected into each of 7 white mice.

4. *Method Presently Employed in Attempts at Isolation From Soils Collected From Field Sources.*—Since the spores appear intimately bound with the soil, the method at present in use in this laboratory consists of injecting mice with the largest amount of soil suspension which they will tolerate without excessive mortality. While a few soil suspensions contain organisms or toxic materials which are fatal to mice, it was found that 1 ml. of a 1 to 10 suspension of most soils could be injected intraperitoneally into stock Swiss mice with a mortality of less than 20 per cent during the first week. The inoculum is prepared as follows: approximately 1 ml. of soil selected at random from a well-mixed soil sample is placed in a sterile test tube and physiological saline solution added to a total of 10 milliliter. Sufficient streptomycin and penicillin are added to make a concentration of 8,000 units of each per milliliter. One milliliter of the resultant mixture is injected intraperitoneally into each of seven 3- to 4-week-old Swiss white mice of the CFW strain. Rarely do more than two of the mice die during the first week. No other antibiotic treatment is given. The mice are autopsied after three to four weeks, and the combined liver and spleen are ground in a mortar. Physiological saline is added to make a suspension sufficient to spread evenly over one blood agar and two Sabouraud's agar plates which are incubated at room temperature.

The results achieved by this method compared to the intermediate method are shown in Table IV.

TABLE IV. EFFICIENCY OF THE FLOTATION METHOD. NUMBER AND PER CENT OF NATURAL SOILS FROM WHICH *Histoplasma* WAS ISOLATED WHEN TESTED BY TWO DIFFERENT METHODS. ALSO SHOWN ARE THE TOTAL NUMBER OF MICE INOCULATED WITH THESE SOILS, AND THE NUMBER AND PER CENT OF MICE POSITIVE BY CULTURE

METHOD	SOILS			MICE		
	NO. TESTED	NO. POSITIVE	PER CENT POSITIVE	NO. INOCULATED	NO. POSITIVE BY CULTURE	PER CENT POSITIVE
Intermediate Method						
Centrifugation of supernatant fluid	103	3	2.9	1274	6	.47
Present Method						
Injection of 1:10 dilution of soil	262	22	8.45	1677	52	3.1

From Table IV it is apparent that approximately 3 per cent of the soils collected from the field and 0.5 per cent of the mice were positive when tested by the *intermediate method.* The so-called *present method,* on the other hand, yielded 8.5 per cent positive soils and 3 per cent positive mice. These results are to be compared with the total of 13 isolations from 676 soils or 1.9 per cent as obtained by the original Emmons' modified method.[4, 5]

DISCUSSION

The results of the experiments reported herein furnished the first experimental evaluation of the flotation method for the isolation of *H. capsulatum* from soil. They indicate that there is no concentration of the spores of this fungus in

the supernatant layers of the extracting fluid, and that the flotation or extraction method is not effective in separating most of the spores from the soil itself. Repeated extraction by shaking and sedimentation apparently did not increase the yield of spores in the extracting fluid. The results were quite similar when either soil freshly inoculated with the spores or soil cultures were employed.

In view of the rather low percentage of positive samples isolated from soil by either Emmons[4] or Zeidberg and associates,[6] it was felt that a more efficient method was needed. Following our earlier results and those of other investigators using the flotation method, we adopted an *intermediate method* employing the sediment of the centrifuged supernatant fluid for the injection of mice. Later experiments resulted in the adopting of the present method which employs a 1 to 10 dilution of the soil itself suspended in antibiotics prior to inoculation of mice. This method appears to be more effective than the previous methods.

The percentage of positive soils obtained by the present method seems rather low. However, many of these soils were collected from areas where we would not expect the fungus to be growing. An analysis of these sources will be published later. The method is presented here only as a possible improvement over presently used methods. It is hoped that eventually a method will be developed which will serve to indicate more accurately the presence of the organism.

Still another point of interest in these experiments is the failure of actidione to inhibit one strain of the fungus in concentrations up to 1.5 mg. per ml. of media. It is possible that actidione may prove useful in differential media for the isolation of *H. capsulatum*, since this antibiotic apparently retards many other fungi. The use of actidione in differential media for the isolation of *C. immitis* has already been reported by Georg and associates.[8]

Although our soil method is still relatively inefficient, it has already proved useful in establishing the etiology of twelve epidemics of pneumonitis by isolation of the organism from the point sources.[9]

Another problem involved in the attempted isolation of the organism from soil is the choice of direct isolation on artificial media or of indirect isolation by the use of animals. Little is known about the relative efficiency of these methods. Direct isolation of *H. capsulatum* from soil has been reported on one occasion.[10] This isolation was obtained after plating over 400 samples on various selective media. Other investigators have attempted to isolate the organism directly from soil without success. These findings suggest that it is neither economical nor desirable to attempt to isolate the fungus directly from soil.

Indirect isolations of the fungus have been made when suspensions of soil were inoculated into animals, usually mice. A number of experiments evaluating the usefulness of these animals in detecting a few spores of *Histoplasma* have been completed. It is sufficient to report, at this time, that a positive culture can usually be obtained when as few as 14 spores are injected into a white mouse by the intraperitoneal method. The data from these investigations will be reported in a separate paper.

SUMMARY

Attempts to concentrate *Histoplasma* spores by the flotation method indicate the relative inefficiency of this method.

The spores appear to be firmly associated with the soil particles, and in our experiments over 90 per cent of them remained in the sedimented soil.

A comparison is made of the efficiency of the various modifications of the flotation method used in this laboratory for the isolation of the organism from soils.

Until a more efficient method is developed, the method of choice appears to be the injection of white mice intraperitoneally with a 1 to 10 dilution of soil previously treated with antibiotics.

A total of 25 isolations from 382 soil samples is reported.

Actidione in concentrations up to 1.5 mg. per milliliter of media failed to inhibit the growth of one strain of *Histoplasma*.

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A NEW METHOD FOR THE DIRECT DETERMINATION OF SERUM CHOLESTEROL

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SINCE Grigaut[1] introduced his procedure for the quantitative estimation of cholesterol utilizing the Liebermann-Burchard reagent[2, 3] developed between 1885 and 1890, numerous modifications of the method have appeared in the literature. Changes in extraction procedures[4-12] and final color development[4-6] for quantitation of cholesterol constitute in the main the various deviations from the earlier method.

Critical discussions and investigations of the various cholesterol methods have been published.[11, 13-16] It would appear that a method which does not involve saponification, extraction, precipitation, drying, and which obtains a stable color of greater sensitivity would be highly desirable. The method presented in this communication obviates the undesirable and time-consuming steps of methods now extant and presents a stable chromogen of far greater sensitivity than that offered by all other published procedures. The method consists of adding a fixed volume of concentrated sulfuric acid, glacial acetic acid, and ferric chloride solution to 0.1 ml. of serum in 3.0 ml. of glacial acetic acid. Full development of the color reaction which is purple requires approximately one minute. The resultant color obeys Beer's law and remains stable over a period of several hours.

METHOD

Reagents.—I. Standard Cholesterol Solution (1 mg. per milliliter): Dissolve 100 mg. of pure, dry, ash-free cholesterol in 100 ml. of 100 per cent glacial acetic acid.

II. Cholesteryl Acetate Solution (1.108 mg. per milliliter). Weigh out 110.8 mg. of pure dry cholesteryl acetate and dissolve in 100 ml. of 100 per cent glacial acetic acid.

III. Ferric Chloride Solution.—Dissolve 10 Gm. of ferric chloride, reagent grade, in 100 ml. of 100 per cent glacial acetic acid.

IV. Color Reagent.—Dilute 2.0 ml. of the ferric chloride solution to 200 ml. with C.P. concentrated sulfuric acid.

Procedure.—(a) *Preparation of the Standard Curve:* Pipette 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of the standard cholesterol solution into clean, dry, 30 ml. test tubes and dilute each with glacial acetic acid to 3.0 milliliter. The acid is measured from a 10 ml. burette. Add 0.1 ml. of distilled water to each standard and mix thoroughly. A blank is prepared which contains 3.0 ml. of glacial acetic acid and 0.1 ml. of distilled water. Pipette in 2.0 ml. of the color reagent by carefully allowing it to flow down the side of the test tube, thus producing two layers. Strike the bottom of the tube sharply while holding it at the top between the thumb and forefinger to effect mixing and even heat distribution. A light brown color first appears which changes to an intense purple within a minute. When the tube has cooled

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to room temperature measure the absorbancy in a spectrophotometer at 560 mμ using a 1 cm. cuvette. The curve obtained when absorbancy is plotted against concentration is shown in Fig. 1, Curve *A*.

(b) *Determination of Total Serum Cholesterol:* Pipette 0.1 ml. of serum into 3.0 ml. of glacial acetic acid in a dry, clean 30 ml. test tube. Add 2.0 ml. of the color reagent and mix as described above. Measure the absorbancy of the solution after it has come to room temperature and determine the total cholesterol content from the calibration curve in Fig. 1.

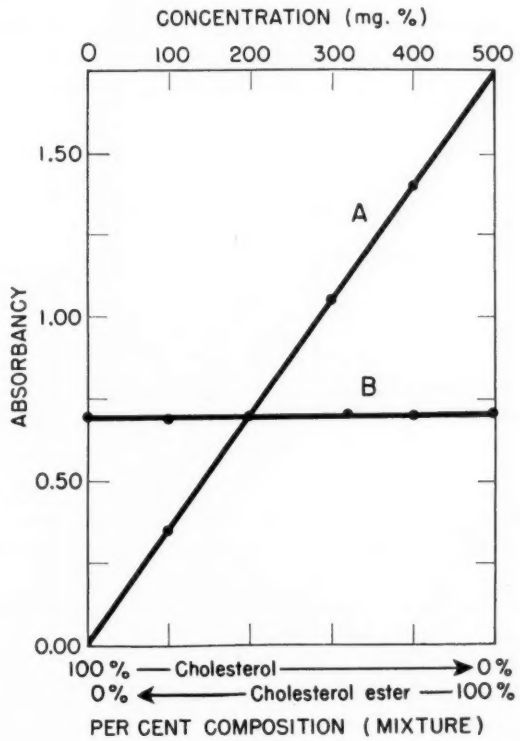

Fig. 1.

DISCUSSION

Fig. 1, Curve *B* illustrates that equimolar concentrations of cholesterol and cholesterol ester yield color of equal intensity. This finding obviates saponification of the ester. The rates of color development with cholesterol or the cholesterol ester appear to be identical. The interference of bilirubin* was studied in some detail. It will be noted in Fig. 2 that only the very high concentrations of this substance in serum will vitiate the cholesterol estimation at 560 mμ. Amounts of 0.1 to 0.8 mg. per cent bilirubin usually encountered in the average serum sample has virtually no effect. The bilirubin absorption curve shown in Fig. 2 illustrates adequately its almost minimal absorbancy at 560 mμ.

Since protein is not removed from the sample a study of its interference was indicated. Curve *A* in Fig. 3 represents the absorption characteristics of

*Eastman Kodak white label.

a 0.1 ml. serum sample (204 mg. per cent as determined by the Schoenheimer-Sperry method) from 400 mμ through 700 mμ following color development by the method described in this investigation. Curve *B* illustrates the color developed by this method with pure cholesterol through the same spectral range. The additive effect of absorbancy permits the subtraction of the pure cholesterol curve absorbancy value from that of the sample containing cholesterol and protein. The results of this measurement are shown in Curve *C* and indicate the negligible effect of protein or other substances present. In summation it may be stated that below 560 mμ one may anticipate a positive error in the determination of cholesterol. Above 560 mμ no such error is in evidence though sensitivity is progressively decreased.

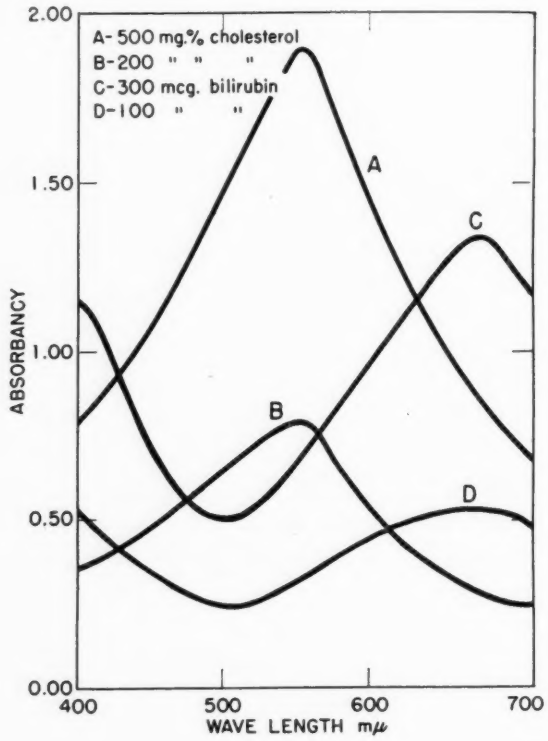

Fig. 2.

A demonstration of the reproducibility and recovery to be expected by this direct method for total cholesterol is shown in Table I. The column labeled mg. per cent present is the average of the ten samples of pooled serum No. 1 in which cholesterol was determined against a standard curve of pure cholesterol. The actual values found are recorded in the column labeled mg. per cent found. Additions were then made to pooled serum No. 1 and the amount actually found and that theoretically present on the basis of the average of the analyses is recorded. The remaining two sera were treated in the same manner. It will

TABLE I. RECOVERIES ON SERUM CHOLESTEROL USING A 0.1 ML. SAMPLE

SAMPLE	CHOL. ADDED (MG. %)	CHOL. PRESENT* (MG. %)	CHOL. FOUND (MG. %)	DEVIATION† (MG. %)
Serum No. 1	0.0	261	258	−3
Serum No. 1	0.0	261	258	−3
Serum No. 1	0.0	261	265	+4
Serum No. 1	0.0	261	262	+1
Serum No. 1	0.0	261	262	+1
Serum No. 1	0.0	261	254	−7
Serum No. 1	0.0	261	250	−11
Serum No. 1	0.0	261	270	+9
Serum No. 1	0.0	261	260	−1
Serum No. 1	0.0	261	268	+7
Serum No. 1	100.0	361	376	+15
Serum No. 1	100.0	361	364	+4
Serum No. 1	100.0	361	370	+9
Serum No. 1	100.0	361	376	+15
Serum No. 1	100.0	361	376	+15
Serum No. 1	100.0	361	365	+4
Serum No. 1	100.0	361	365	+4
Serum No. 1	100.0	361	360	−1
Serum No. 1	100.0	361	368	+7
Serum No. 1	100.0	361	365	+4
Serum No. 2	0.0	210	206	−4
Serum No. 2	0.0	210	216	+6
Serum No. 2	0.0	210	208	−2
Serum No. 2	100.0	310	308	−2

*The amount present is based on the arithmetical mean calculated from the values obtained on ten separate samples.

†Mean deviation of a single observation is equal to ± 5.8 milligrams per cent.

TABLE II. COMPARISON OF DIRECT METHOD WITH EXTRACTION PROCEDURES

SAMPLE	EXTRACTION	CHOL. ADDED (MG. %)	CHOL. FOUND (MG. %)
P.S No. 1	none	0.0	238
P.S No. 1	none	0.0	248
P.S No. 1	alcohol-acetone	0.0	248
P.S No. 1	alcohol-acetone	0.0	245
P.S No. 1	alcohol-acetone	0.0	248
P.S No. 1	alcohol-acetone	0.0	248
P.S No. 1	alcohol-acetone	100.0	347
P.S No. 1	alcohol-acetone	100.0	357
P.S No. 1	alcohol-acetone	100.0	360
P.S No. 1	alcohol-acetone	100.0	357
P.S No. 1	alcohol-acetone	100.0	353
P.S No. 2	none	0.0	268
P.S No. 2	alcohol-acetone	0.0	258
P.S No. 3	none	0.0	210
P.S No. 3	alcohol-acetone	0.0	209
P.S No. 4	none	0.0	192
P.S No. 4	alcohol-acetone	0.0	180
P.S No. 5	none	0.0	153
P.S No. 5	alcohol-acetone	0.0	150
P.S No. 6	none	0.0	320
P.S No. 6	alcohol-acetone	0.0	302
P.S No. 7	none	0.0	207
P.S No. 7	none	0.0	185
P.S No. 7	none	0.0	208
P.S No. 7	Chloroform	0.0	208
P.S No. 8	none	0.0	197
P.S No. 8	none	100.0	280
P.S No. 8	acetic acid	0.0	188
P.S No. 8	alcohol-acetone	0.0	185

be noted that both reproducibility and recoveries are well within acceptable analytical standards.

Table II shows a comparison of results for total cholesterol made directly on pooled serum with those obtained on the extract of the same serum using the Schoenheimer-Sperry alcohol-acetone mixture. Recovery studies were also made on serum No. 1 by additions of pure cholesterol to the serum extract. Results obtained by the direct method after the additions and those using the direct method on the Schoenheimer-Sperry extract are listed. In some instances the results obtained on the extract appeared to be lower than the usual precision that this method yields. This was attributed to incomplete extraction. All results were considered, however, to be within close enough agreement to be valid and acceptable of interpretation for investigative purposes.

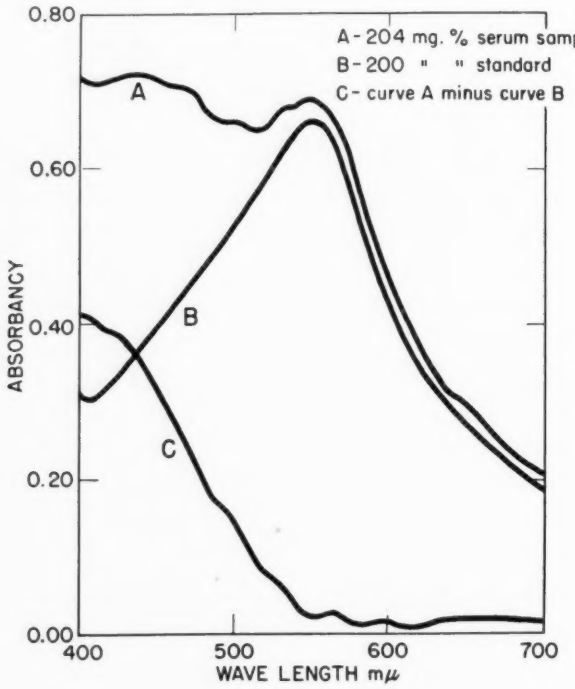

Fig. 3.

Finally a comparison of the direct method with Schoenheimer-Sperry and Kingsley-Schaffert procedures was made (Table III). Agreement here is within analytical license. It will be noted that in general the results of the direct method are somewhat higher. This is attributed to the necessity of employing several steps in the other methods as compared to the method described in this manuscript.

Earlier, under reagents, the authors have specified the use of 100 per cent acetic acid. In this laboratory we have used both 100 per cent and 99.5 per

TABLE III

SAMPLE	COMPARISON METHOD	CHOL. FOUND (X)	CHOL. FOUND (DIRECT) (Y)	X-Y
A	K.S*	280	285	−5
B	K.S	234	232	+2
C	K.S	228	215	+13
D	K.S	206	208	−2
E	S.S	186	188	−2
F	S.S	260	268	−8
G	S.S	120	126	−6
H	S.S	170	182	−8
I	S.S	266	268	−2
J	S.S	220	210	+10
K	S.S	180	192	−12
L	S.S	152	145	+7
M	S.S	152	152	0
N	S.S	280	280	0
O	S.S	310	316	−6
P	S.S	206	197	+9
Q	S.S	227	213	+14
R	S.S	270	295	−25
S	S.S	219	203	+16
T	S.S*	183	190	−7

*Average of four samples.

cent acetic acid. It appears that the only impurity which might interfere and which occasionally occurs in acetic acid is glyoxylic acid.[17] This compound will give a color with the tryptophane present in serum. To date this has not been the occasion for difficulty in our hands. The reagents as described are stable and last for weeks.

SUMMARY

A direct method for the quantitative determination of total serum cholesterol is described. The procedure consists of adding a reagent containing sulfuric acid, acetic acid, and ferric chloride to a 0.1 ml. sample of serum. The sensitivity achieved is several times that of current procedures and the time required for a single determination is in the order of minutes. Precision and reproducibility are demonstrated as well as the absorption characteristics of the purple color produced through the spectral range of 400 to 700 mμ. Finally, the method is compared with samples in which total cholesterol is determined by the Kingsley-Schaffert and Schoenheimer-Sperry procedures.

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DEVICE FOR COLLECTION OF HUMAN PAROTID SALIVA

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THE collection of human parotid saliva was first achieved by Ordenstein[1] in 1860 through cannulation of Stenson's duct. Similar methods were employed by Eckhard[2] and Oehl.[3] Since this procedure was neither accurate nor comfortable to the patient,[4] investigators during this century have usually employed suction devices to cap the outlet of Stenson's duct. The first "suction caps" were constructed with the suction applied to the duct itself. Later modifications of suction caps obtained retention of the cap by means of suction applied to a ring of tissues surrounding the duct. The latter type included the devices by Lashley,[5] Krasnogorski,[6] Finesinger and Finesinger,[7] and Jenness and Hackman.[8] These procedures were employed by Richter and Wada,[9] Winsor,[10-12] and Lourie[13] for measuring rate of flow of parotid saliva.

Investigators associated with the author had need for a device which could be sterilized and used for a number of patients without causing discomfort, which would avoid contamination with other oral secretions, and which could be adaptable for measuring the rate of flow. The device finally developed seems to fulfill these requirements.

Transparent plastic was selected as material for the suction cap and the tubing. The size of the collection chamber which covers Stenson's duct and the annular suction ring was investigated. The minimum depth of the collection chamber was found to be 3 mm. About 6 mm. was suitable for the diameter of the collection chamber. The retention of the cap over the duct was excellent when the annular ring surrounding the collection chamber was 2 mm. deep and 1.5 mm. wide. Fig. 1 shows a side and bottom view of the suction cap that is machined from methyl methacrylate polymer. The tubing employed is number 205 polyethylene tubing.* The entrance holes are reamed to size and the tubing is pressed into place.

The complications of some of the other devices have been overcome simply by supplying suction by means of a small rubber pipette ball fixed to the hilt of a No. 15 hypodermic needle. The suction developed by the bulb is similar to that recommended by Lourie.

Previous systems have had a single tube leading to the collection chamber. With this design air bubbles cannot be flushed from the system. Bubbles can be eliminated simply by arranging for two entrances to the collection chamber, through one of which a stream of water or disinfectant is introduced prior to collection.

From the Laboratory of Oral Physiology, Tufts College Dental School.
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*Supplied by the Clay-Adams Company, New York, N. Y.

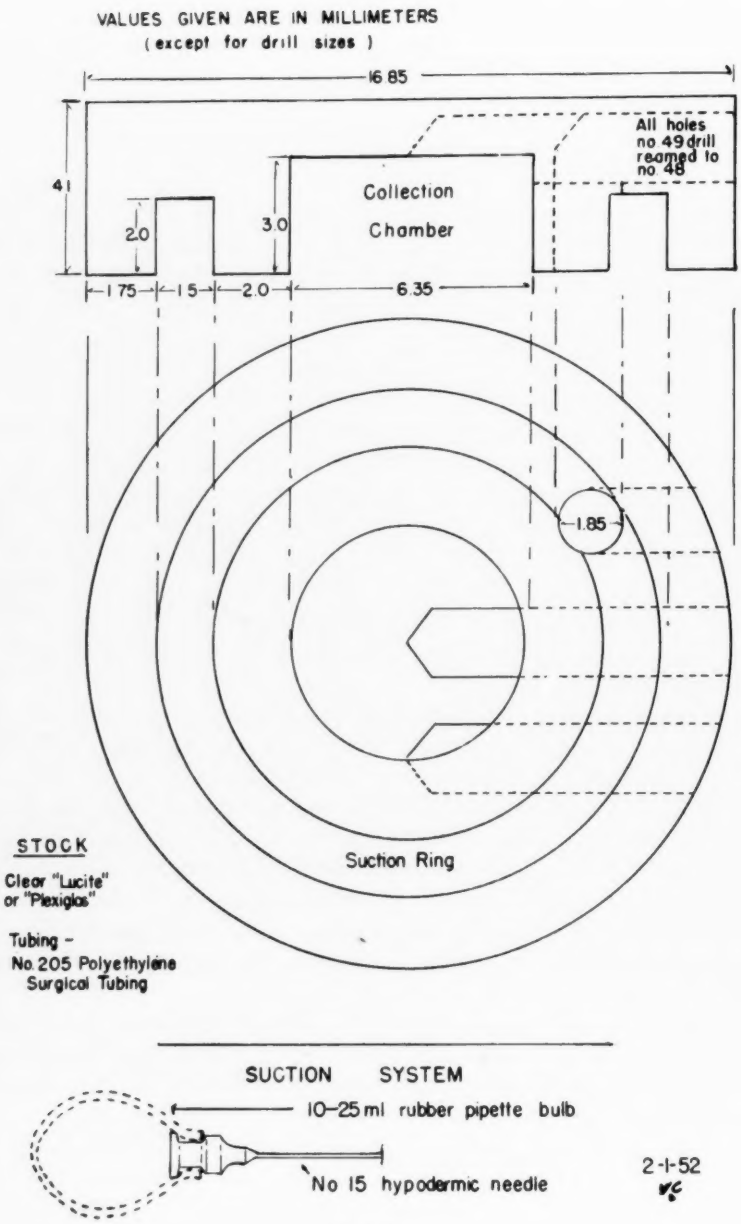
VALUES GIVEN ARE IN MILLIMETERS
(except for drill sizes)
16.85
All holes no. 49 drill reamed to no. 48
4.1
2.0
3.0
Collection Chamber
1.75
1.5
2.0
6.35
1.85
Suction Ring
STOCK
Clear "Lucite" or "Plexiglas"
Tubing –
No. 205 Polyethylene Surgical Tubing
SUCTION SYSTEM
10–25 ml rubber pipette bulb
No. 15 hypodermic needle
2-1-52
VC

Fig. 1.

The suction cap is stored before use in a 1 to 1000 Zephiran Chloride solution for eighteen hours, and rinsed in sterile distilled water with three changes for one hour. Stenson's duct is washed with 70 per cent alcohol on a gauze pad. The suction bulb is squeezed, the cap is centered over Stenson's duct, and the bulb is released. The collection chamber is flushed with 70 per cent alcohol followed by sterile distilled water. For flow studies, the collection chamber is merely filled with water, but for collecting samples for analysis the water is

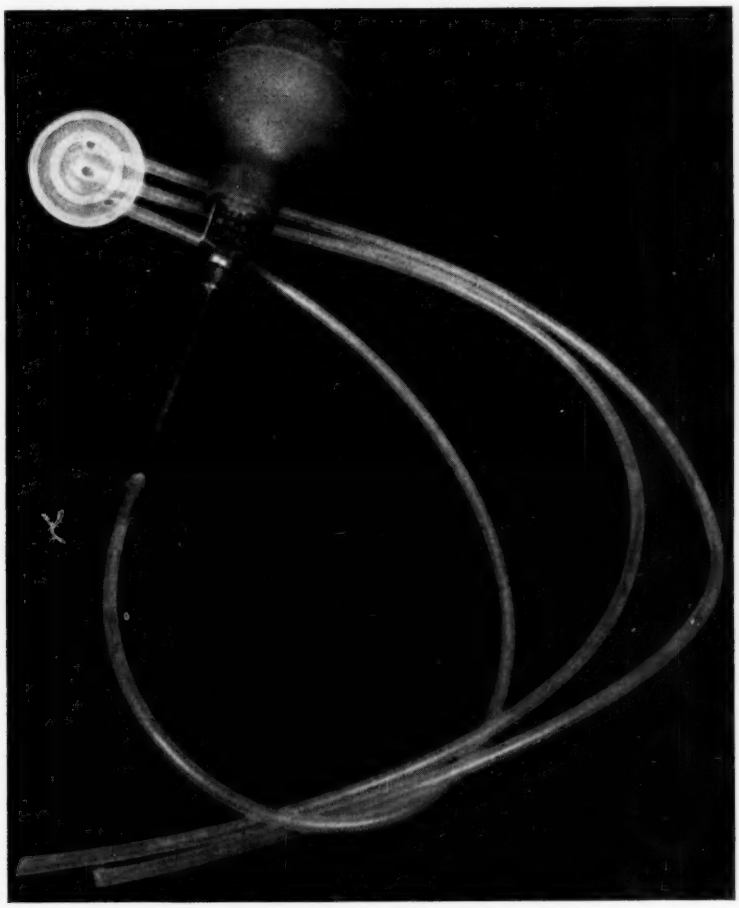

Fig. 2.

blown out prior to collection. Both parotid ducts can be capped simultaneously without causing inconvenience during chewing and without preventing the collection of sublingual and submaxillary saliva. Fig. 2 shows the complete assembly. When the device is to be removed, it is well to release the suction and let the cap remain in position in the mouth for several seconds, in order to allow cheek tissue to return to its normal position. This precaution obviates pulling or cutting tissue when the device is removed. It should also be noted that long con-

tact with 70 per cent alcohol will soften and crack the plastic and this type of sterilization should be avoided.

SUMMARY

Specifications are provided for a device for collecting human parotid saliva. The equipment has been designed to make it easy to construct, to sterilize, and to wear.

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A NEW METHOD FOR PREPARING A HIGHLY PURIFIED URICASE SUITABLE FOR URIC ACID DETERMINATION BY ULTRAVIOLET SPECTROPHOTOMETRY

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IN OUR work on purine metabolism we have used Praetorius's adaptation[1] of Kalckar's method[2] for determining uric acid. This method, which combines the use of a highly purified uricase and differential ultraviolet spectrophotometry, is the most specific, accurate, and quickest procedure thus far devised for measuring uric acid in biological fluids. The disadvantages of the method are the length of time required for the purification of uricase, the tedious preparation of the aluminum hydroxyd-C-γ, and the instability of the enzyme.[3]

It is the purpose of this paper to outline a short and simple procedure for the purification of uricase in the hope that it will result in wider use of Kalckar's specific and accurate method of uric acid determination. Experience to date indicates that the enzyme so prepared is very stable when kept under proper conditions; one lot can be used for two hundred determinations.

METHOD OF PREPARATION

In order to obtain better yields and to permit the use of smaller and more convenient volumes, it was found necessary to prepare the acetone powder in the following manner:

1. Fresh pig's liver is stored on ice overnight. The next day 600 Gm. of liver are put through a meat grinder, quickly homogenized in a Waring blender with 2000 ml. of −10° C. acetone, stirred for ten minutes, following which the acetone is sucked off through a large Büchner funnel. The residue is again homogenized with 2000 ml. of −10° C. acetone, stirred for ten minutes, and the acetone sucked off through the Büchner funnel. The partly dry residue is then passed through a No. 20 sieve, and the resulting fine powder is used for extraction. The above procedure should be carried out at −5° C. or less and the powder should be kept at this temperature. It is not necessary to dry the powder in a desiccator.

2. Twenty-five grams of the acetone powder are washed at 4° C. with 250 ml. of precooled 0.1M. phosphate buffer, pH 7.3. In order to obtain optimal extraction of inactive proteins it is most important to have the acetone powder homogeneously suspended in the buffer and stirred for at least fifteen minutes. After centrifugation the supernatant solution is analyzed for protein content using Lowry's formula.[2]

Protein concentration in mg./ml. $= 1.45 \times E_{280m\mu} - 0.74 \times E_{260m\mu}$. The supernatant solution should contain at least 15 mg. protein per milliliter. To obtain this concentration of protein a second phosphate buffer washing may be required.

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3. The residue is extracted for twenty minutes at 40° C. with 500 ml. 0.1M borate buffer, pH 10.0 After the addition of 20 ml. of 50 per cent barium acetate, the extract is centrifuged and the insoluble material discarded. Ten milliliters of a neutralized, saturated solution of ammonium sulfate is mixed with the supernatant solution; the barium sulfate formed is removed by centrifugation.

4. The clear, yellow-red, supernatant solution is cooled to 0° C., and then mixed with an equal volume of ice-cold, neutralized, saturated solution of ammonium sulfate. The precipitate formed is allowed to stand for a few mintues, centrifuged off, and redissolved in 25 ml. of cold distilled water. Then a neutralized, saturated solution of ammonium sulfate is added to give a final saturation of 10 per cent. This solution is kept overnight at 4° C.

5. The enzyme-containing material comes down as a brown precipitate which is centrifuged off at 16,000 r.p.m. and immediately extracted, first with 2.5 ml., and then with 1.5 ml. of 0.2M. acetate buffer, pH 4.8. The dark residue is discarded after centrifugation. The water-clear extracts are neutralized with 1M. glycine buffer, pH 9.4. The neutralization should be done as quickly as possible in order to minimize the loss of enzyme activity while in the acid medium. A white precipitate is formed on centrifugation which is brought into suspension with 2.0 ml. of 10 per cent saturated ammonium sulfate solution.

One hundredth milliliter of this suspension with an enzymatic activity of at least 100γ per minute per milliliter is used per determination. The enzymatic activity is measured by pipetting 0.01 ml. of the uricase solution into a Beckman quartz cell containing a uric acid solution of 5γ per milliliter. The change in extinction at 290 mμ in one minute over the constant part of the decay curve is measured. If the enzyme is kept in a frozen state it retains its activity for at least three months.

SUMMARY

A much simpler, and considerably less time-consuming modification of Holmberg's method for the preparation of a highly purified, stable uricase preparation has been described. It is hoped that the introduction of this method of preparing uricase will result in more widespread use of Kalckar's specific, accurate and quick method for the determination of uric acid in biological fluids.

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